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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/678,723	10/02/2003	Eduardo Marban	001107.00459	6122

7590 11/16/2006

Robert L. Buchanan
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OIPE IAP88
DEC 04 2006
PATENT & TRADEMARK OFFICE

EXAMINER
MONTANARI, DAVID A

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 11/16/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

BEST AVAILABLE COPY

Office Action Summary	**Application No.** 10/678,723	**Applicant(s)** MARBAN ET AL.	
	Examiner David Montanari	**Art Unit** 1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on 24 August 2006.
2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.
3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-75 is/are pending in the application.
4a) Of the above claim(s) 5,8-19,40,43-45 and 49-75 is/are withdrawn from consideration.
5)☐ Claim(s) _____ is/are allowed.
6)☒ Claim(s) 1-4,6,7,20-39,41,42 and 46-48 is/are rejected.
7)☐ Claim(s) _____ is/are objected to.
8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.
10)☒ The drawing(s) filed on 02 October 2003 is/are: a)☒ accepted or b)☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
11)☒ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a)☐ All b)☐ Some * c)☐ None of:
1.☐ Certified copies of the priority documents have been received.
2.☐ Certified copies of the priority documents have been received in Application No. _____.
3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

1. Applicant's election without traverse of Group I claims 1-4, 6, 7, 20-39, 41, 42, and 46-48 in the reply filed on 8/24/2006 is acknowledged.

2. Claims 5, 8-19, 40, 43-45, and 49-75 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected subject matter, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 8/24/2006.

3. Claims 1-4, 6, 7, 20-39, 41, 42, and 46-48 are examined in the instant application.

Oath/Declaration

The oath or declaration is defective. A new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. See MPEP §§ 602.01 and 602.02.

The oath or declaration is defective because:

> Non-initialed and/or non-dated alterations have been made to the oath or declaration. See 37 CFR 1.52(c).
>
> It does not identify the city and either state or foreign country of residence of each inventor. The residence information may be provided on either an application data sheet or supplemental oath or declaration.

Claim Rejections - 35 USC § 112

Claims 1-4, 6, 7, 20-39, 41, 42, and 46-48 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of modulating calcium channel activity in a rodent heart comprising administering locally to the heart an adenovirus comprising a nucleic acid that encodes the human guanosine triphosphatase protein (GEM), does not reasonably provide enablement for a method of modulating calcium channel activity in any mammalian heart. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

While determining whether a specification is enabling, one considers whether the claimed invention provides sufficient guidance to make and use the claimed invention, if not, whether an artisan would have required undue experimentation to make and use the claimed invention and whether working examples have been provided. When determining whether a specification meets the enablement requirements, some of the factors that need to be analyzed are: the breadth of the claims, the nature of the invention, the state of the prior art, the level of one of ordinary skill, the level of predictability in the art, the amount of direction provided by the inventor, the existence of working examples, and whether the quantity of any necessary experimentation to make or use the invention based on the content of the disclosure is "undue" (In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)). Furthermore, USPTO does not have laboratory facilities to test if an invention will function as claimed when working examples are not disclosed in the specification, therefore, enablement issues are raised and discussed based on the state of knowledge pertinent to an art at the time of the invention, therefore skepticism raised in the enablement rejections are those raised in the art by artisans of expertise.

The breadth of the claims encompasses modulating the calcium channel activity in any mammalian heart. This embodiment reads on gene therapy.

Whereas the nature of the invention is a method to control heart contractility in rodent models of heart failure, a review of the current art teaches that the field of gene therapy is unpredictable. At the time the invention was made, successful implementation of gene therapy protocols was not routinely obtainable by those skilled in the art. This is reflected by three reviews. Concalves (2005, BioEssays, Vol. 27, pgs. 506-517) teaches that with respect to gene therapy "one can conclude that further improvements in gene transfer technologies (e.g. control over transgene expression and integration) and deeper insights in host-vector interactions (e.g. knowledge on vector and gene-modified cell biodistribution following different routes of administration and the impact of innate and adaptive immunity) are warranted before clinical gene therapy reaches maturity" (pg. 514 col. 2 parag. 3). Specifically, Concalves teaches that gene therapy utilizing viruses has resulted in significant unpredictability, with retroviruses (pg. 513 col. 1 parag. 3 bridge col. 2 parag. 1), and adenoviruses (pg. 513, col. 2 parag. 2) providing only a fleeting success that was ultimately undone by a lack of transduction and low transgene expression levels. Parekh-Olmedo et al. teach (2005, Gene Therapy, Vol. 12, pgs. 639-646) with regard to gene repair utilizing gene therapy comprising single-stranded DNA oligonucleotides, repair of a gene of interest is again unpredictable. Specifically, successful gene repair has been reported in an animal model of severe kidney disease comprising carbonic anhydrase-deficiency utilizing a single-stranded DNA vector (pg. 641 col. 1 parag. 3). However, in a LacZ mouse model designed for testing gene repair using a variety of vectors, no correction of the mutant LacZ gene was observed, resulting in the observation that delivery of DNA to a particular tissue continues to be a problem (pg. 641 col. 2 parag. 1). Verma et al. (2005, Annu. Rev. Biochem. Vol. 74, pgs. 711-738) teach that "the young field of gene therapy promises major medical progress toward a cure of a broad spectrum of human diseases from immunological disorders to heart disease and cancer. It has, therefore, generated great hopes and great hypes, but it has yet to deliver its promised potential" (pg. 732, parag, 2 lines 2-6). Verma et al. continues to teach that the process of gene delivery and expression is known as transduction, and that successful transduction requires overcoming a number of obstacles that are common to all vector systems (pg. 712, parag. 2 lines 1-3). Specifically, these obstacles include production of the vector, the targeting of the vector to a specified cell type, sustained gene expression, and avoidance of potential hazards such as insertional mutagenesis and immune responses (pg. 712, parag. 2 lines 3-19). With regard to targeting the heart via gene therapy, the art teaches that specific intrinsic challenges exist when the skilled artisan attempts to target the heart and the cardiovascular system (Fishbein et al., 2005, J. Controlled Release, Vol. 109, pgs. 37-48). Fishbein et al. teaches that specifically, delivery challenges to the heart are one of the primary roadblocks to successful treatment in the heart (pg. 47, col. 1).

In view of the unpredictability of the art of gene therapy, a skilled artisan would require specific guidance in the instant disclosure to make and use the full scope of the claimed embodiment. Wherein the instant specification provides specific guidelines for delivering adenovirus locally to the rodent heart, has not provided any relevant teachings, guidance, or working examples that teach or otherwise correlate to local delivery of a adenovirus with consummate expression of GEM protein in any mammalian heart. Thus in view of the lack of guidance and direction provided by the specification for gene therapy, it would have required one of skill in the ad undue experimentation to make and use the invention as claimed.

The working examples provided in the instant specification teach that adenovirus comprising a nucleic acid encoding GEM was locally delivered to guinea-pig hearts (Example 1, pg. 41). The specification continues to teach that GEM expression results in inhibition of L-type calcium current (Example 1, pg. 41), shortened the QT interval (Example 2, pgs. 44), and reduction of heart rate in atrial fibrillation (Example 3, pg. 45) in guinea-pigs as well as attenuation of cardiac hypertrophy (Example 4, pg. 46) in mice. However, the specification has failed to teach the modulation of calcium channel activity in any mammalian heart. As discussed above, the art teaches that significant unpredictability exists with regard to the administration of adenoviruses. Therefore, in view of the lack of direction or guidance provided by the instant specification for gene therapy using the claimed method, the quantity of experimentation necessary to determine the parameters listed above for producing a successful delivery and expression of GEM in any mammalian heart, the absence of working examples in other species of mammals, and the unpredictability of the art of gene therapy with respect to obtaining any effect on any symptom of any disease or condition, it would have required undue experimentation for one skilled in the art to make and/or use the claimed invention.

Therefore, in view of the breadth of the claims and the lack of guidance provided by the specification as well as the unpredictability of the art, the claimed invention is not enabled for its full breadth and limiting the scope of the claimed invention to a method of modulating calcium channel activity in a rodent heart comprising administering locally to the heart an adenovirus comprising a nucleic acid that encodes the mammalian GEM is proper.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 7, 20, and 30 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 7 is unclear. In line two the phrase “by at least about 10%” is stated. It is unclear what value is needed to properly reference the reduction of the number of L-type calcium current channels.

Claim 20 recites the limitation "wherein the recombinant animal virus" in reference to claim 1. There is insufficient antecedent basis for this limitation in the claim.

Claim 30 is unclear. It states that the recombinant adenovirus have a molecular weight between about 35 kilobases to about 40 kilobases. Kilobases are not a measure of weight, but of nucleotide length. Appropriately, kilodaltons at this molecular level are commonly used.

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to David Montanari whose telephone number is 1-571-272-3108. The examiner can normally be reached on M-Tr 8-6.

If attempts to reach the examiner by telephone are unsuccessful, the examiner’s supervisor, Peter Paras can be reached on 1-571-272-4517. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

David A. Montanari, Ph.D.

DAVE TRONG NGUYEN
SUPERVISORY PATENT EXAMINER

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Notice of References Cited	Application/Control No. 10/678,723	Applicant(s)/Patent Under Reexamination MARBAN ET AL.
	Examiner David Montanari	Art Unit 1632
		Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
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	N					
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	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Concalves M, A concise peer into the background, initial thoughts, and practices of human gene therapy, 2005, BioEssays, Vol. 27, pgs. 506-517.
	V	Parekh-Olmedo H, Gene therapy progress and prospects: targeted gene repair, 2005, Gene Therapy, Vol. 12, pgs. 639-646.
	W	Verma IM, Gene Therapy: Twenty-first century medicine, 2005, Annu. Rev. Biochem. Vol. 74, pgs. 711-738.
	X	Fishbein I, Site specific gene delivery in the cardiovascular system, 2005, J. Controlled Release, Vol. 109, pgs. 37-48.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

U.S. Patent and Trademark Office
PTO-892 (Rev. 01-2001) **Notice of References Cited** Part of Paper No. 20061109

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	10/678,723
Filing Date	October 2, 2003
First Named Inventor	Eduardo Marban
Art Unit	1632
Examiner Name	~~TBA~~ McKinnon, D.
Attorney Docket Number	001107.00459 /

U.S. PATENT DOCUMENTS

Examiner Initials *	Cite No.[1]	Document Number Number - Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
DM		US-5,436,128	07-25-1995	Harpold et al	
		US- 5,824,550	10-20-1998	Hruska et al	
		US- 5,965,396	10-12-1999	Pan et al	
		US- 6,156,726	12-05-2000	Newcomb et al	
		US- 6,365,337	04-02-2002	Letts et al	
		US- 2002/0009772 A1	01-24-2002	Snutch	
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		US- 2003/0118988 A1	06-26-2003	Kandel et al	
		US- 6,605,274 B1	08-12-2003	Dillmann et al	
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Examiner Signature		Date Considered	11/9/06

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Sheet 2 of 2

Complete if Known	
Application Number	10/678,723
Filing Date	October 2, 2003
First Named Inventor	Eduardo Marban
Group Art Unit	1632
Examiner Name	~~To Be Assigned~~ Montanari, D.
Attorney Docket Number	001107.00459

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
DM		MARBAN, "Cardiac Channelopathies", Nature, January 10, 2002, pp. 213-218, Vol. 415.	
		MAGUIRE et al., "Gem: An Induced, Immediate Early Protein Belonging to the Ras Family", Science, July 8, 1994, Vol. 265.	
		LEONE et al., "The Gem GTP-binding Protein Promotes Morphological Differentiation in Neuroblastoma", Oncogene, pp. 3217-3225, Vol. 20.	
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		MARBAN et al., "Gene Therapy for Cardiac Arrhythmias", Cold Spring Harbor Symposia on Quantitative Biology, 2002, Vol. LXVII.	
		MIAKE et al., "Biological Pacemaker Created by Gene Transfer", Nature, September 12, 2002, pp. 132-133, Vol. 419.	
		DOLPHIN, "Mechanisms of Modulaton of Voltage-Dependent Calcium Channels by G Proteins", Journal of Physiology, 1998, pp. 3-11, Vol. 506.1.	
		ERTEL, "Nomenclature of Voltage-Gated Calcium Channels", Neuron, March, 2000, pp. 533-535, Vol. 25.	
		BEGULN et al., "Regulation of Ca^{2+} Channel Expression at the Cell Surface by the Small G-protein kir/Gem", Nature, June 7, 2001, pp. 701-706, Vol. 411.	
		CATTERALL, "Structure and Regulation of Voltage-Gated Ca^{2+} Channels", Annual Rev. Cell Dev. Biology, 2000, pp. 521-55, Vol. 16.	
		WALKER et al., "Subunit Interaction Sites in Voltage-Dependent Ca^{2+} Channels: Role in Channel Function", Elsevier Science, Ltd.; 1998, pp. 148-154, Vol. 21.	
		TRIMMER, "Unexpected Cross Talk: Small GTPase Regulation of Calcium Channel Trafficking", Science's Stke, January 8, 2002, pp. 1-3.	

Examiner Signature		Date Considered	11/9/06

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Sheet 1 of 1

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First Named Inventor	Eduardo Marban
Art Unit	1632
Examiner Name	~~TBA~~ Montanez, DA
Attorney Docket Number	001107.00459

U.S. PATENT DOCUMENTS

Examiner Initials *	Cite No.[1]	Document Number Number - Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
DM		US- 6,214,620	04/10/2001	Johns et al	
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Sheet 1 of 1

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Art Unit	~~TBA~~ 1632
Examiner Name	~~TBA~~ Montanari
Attorney Docket Number	001107.00459

NON PATENT LITERATURE DOCUMENTS

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DM		ARESTA, S. et al., "A Novel Rho GTPase-activating-protein interacts with Gem, a member of the Ras superfamily of GTPases," *Biochem. J.*, 2002, pp. 57-65, Vol. 367	
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Site specific gene delivery in the cardiovascular system

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Abstract

Gene therapy holds great promise for treating both genetic and acquired disorders. However, progress toward effective human gene therapy has been thwarted by a number of problems including vector toxicity, poor targeting of diseased tissues, and host immune and inflammatory activity to name but a few of the challenges. Gene therapy for cardiovascular disease has been the subject of many fewer clinical trials than other disorders such as cancer or cystic fibrosis. Nevertheless, the challenges are comparable. The present paper reports a review of investigations related to our hypothesis that site specific cardiovascular gene therapy represents an approach that can lead to both optimizing efficacy and reducing the impact of gene vector-related systemic adverse effects. We report experimental studies demonstrating proof of principle in three areas: gene therapy for heart valve disease, gene delivery stents, and gene therapy to treat cardiac arrhythmias. Heart valve disease is the second most common indication for open heart surgery and is now only treatable by surgical removal or repair of the diseased heart valve. Our investigations demonstrate that gene vectors can be immobilized on the surface of prosthetic heart valve leaflets thereby enabling a therapeutic genetic modification of host cells around the valve annulus and on the leaflet. Other animal studies have shown that vascular stents used to relieve arterial obstruction can also be used as gene delivery systems to provide therapeutic vector constructs that can both locally prevent post stenting reobstruction, known as in-stent restenosis, and treat the underlying vascular disease. Cardiac arrhythmias are the cause of sudden death due to heart disease and affect millions of others on a chronic basis. Our group has successfully investigated in animal studies localized gene therapy using an ion channel mutation to treat atrial arrhythmias.

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1. Introduction

Cardiovascular disease is the most common cause of death and morbidity in developed countries. Cardiovascular disease is in general focal and developments in diagnostic techniques have made it possible to localize and even access via

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surgery or cardiac catheterization disease sites in the cardiovascular system. Thus, although there are systemic medications for some cardiovascular diseases, interventions have played a large role in recent times by delivering cardiovascular devices or device related therapies to localized lesions. For the past decade the advent of stent angioplasty, and the even more recent use of drug eluting stents has resulted in a paradigm shift in the care of coronary artery disease and post-stenting arterial re-obstruction known as in-stent restenosis (ISR). Gene therapy for cardiovascular disease, while showing great promise in experimental studies, has not been used extensively in human clinical trials. Three site specific gene delivery strategies for the cardiovascular system will be reviewed in this paper: gene delivery stents, gene therapy for heart valve disease, and gene therapy for cardiac arrhythmias.

2. Materials and methods

2.1. Gene delivery stent methods

2.1.1. Bulk immobilization of plasmid vectors in PLGA or denatured collagen stent coatings

A solution of plasmid DNA (10 mg/ml in 0.05 M Tris/EDTA, pH 7.4) encoding for green fluorescent protein (GFP) was added into a solution of polylactic–polyglycolic acid copolymer (PLGA) *Mr* 50,000 in chloroform while vortexing on ice. The resulting emulsion was coated in multiple thin layers onto 15 mm Crown (Cordis, Warrren, NJ) stents resulting in a configuration of a uniform polymer layer incorporating DNA in dispersed form [1]. Similarly, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)-activated heat-denatured collagen was mixed with GFP plasmid DNA followed by 3–5 applications of the viscous solution onto stents [2] with drying periods at 37 °C under vacuum. Collagen-coated stents were finally dipped briefly in PLGA/chloroform to obtain sealing layer preventing rapid DNA release from collagen-coated stents [2].

2.1.2. Antibody-mediated surface immobilization of adenoviral vectors on collagen stent coating

Bovine type I collagen was neutralized to pH 7.4 followed by the addition of cross-linking agent (EDAC; 0.1 mg/mg collagen), and the solution was incubated at 37 °C for 10 min prior to coating of the stents mounted on rotating platform as previously published [3]. The collagen-coated stents were exposed to a solution of the bifunctional amine/thiol-reactive cross-linker, *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, 20 mg/ml) in DMSO:PBS (2:1 v/v), and incubated for 2 h at room temperature. After rinsing with copious PBS, the SPDP-activated stents were put into a solution of an anti-knob $(Fab)'_2$ antibody (5 μg in 100 μl). A type 5 replication defective adenovirus (Ad) encoding GFP (Ad-GFP) was used as a model vector. The antibody bound collagen-coated stents were added to Ad-GFP suspension in PBS and incubated at 37 °C for 1 h followed by extensive PBS rinsing to remove any unbound virus. For in vitro characterization, a dosage range of 10^7–10^{10} viral particles/mg collagen was used. For animal implant studies, the Ad-GFP loading dosage was 5×10^{10} viral particles/stent. Collagen films in culture dishes were formulated on the surfaces of culture plates and activated as above, but in situ on the dish surface.

2.2. Heart valve methods

2.2.1. Adenovirus constructs and polyurethane configurations thiolated polyurethane for adenovirus tethering

A replication defective adenovirus, Ad-GFP, was tethered to polyurethane using previously published techniques [4], Briefly, Tecothane® (Thermedics, Waltham, MA) TT1074A was dissolved in *N,N*-dimethyacetamide (DMAc) and bromoalkylated with a large excess of 1,4-dibromobutane in the presence of lithium tert-butoxide. Acetylated thiol groups were introduced to the bromoalkylated polymer by incubating the polymer with thioacetic acid under mildly basic conditions. The polyurethane was cast into 0.15 mm thick films. To generate thiol groups, the polyurethane films were reacted with 5 M aqueous NH_3. The molecular cross linker, 1,4-Di-[3′-(2′-pyridyldithio)-propionamido] butane, was added to activate binding sites for an antiknob (Fab)'2 antibody generated in a mice. The films were then incubated overnight with 10^8 PFU/ml of Ad-GFP.

2.2.2. Cholesterol modified polyurethane for optimal cell adhesion

Cholesterol was covalently bound to the urethane hard segments by reacting bromobutylated Tecothane® with 2-hydroxy-3-β-cholesteryloxypropanethiol as previously described [5]. Briefly, glycidyl cholesterol was prepared by dissolving cholesterol in DMAc and reacting with lithium tert-butoxide followed by the addition of epibromohydrin. Glycidyl cholesterol was reacted with thioacetic acid to yield 1-acetylthio-3-β-cholesteryloxy-2-propanol which was dissolved in tetrahydrofuran and mixed with a 2.7M solution of NH_2OH to yield 2-hydroxy-3-β-cholesteryloxypropanethiol.

2.2.3. Blood outgrowth endothelial cell (BOEC) harvesting

Sheep BOECs were prepared from freshly drawn blood using a previously published method [6]. Briefly, heparanized peripheral blood (100 ml) was obtained from juvenile sheep via venipuncture. Mononuclear cells were separated from the blood using Histopaque 1077 (Sigma, St. Louis, MO). The buffy coat was collected, washed with Hank's balanced Salt Solution and resuspended with EGM2 culture media (BioWhitaker, Walkersville, MD). The resuspended cells were seeded on tissue culture dishes precoated with acetic acid denatured collagen. The culture media was changed every other day and the cells were subcultured using trypsin/ethylenediaminetetraacetic acid. Endothelial cell phenotype was confirmed using uptake of acetylated low density lipoprotein, immunocytochemistry for P1H12 and VE-cadherin.

2.2.4. Sheep surgery

Surgery was performed on juvenile sheep (4–5 months of age) by the Cardiovascular Surgical Laboratories at the University of Minnesota. Briefly, sheep were anesthetized using halothane/isofluorane. An approximately 1.5 cm^2 polyurethane valve leaflet was fashioned during surgery from polyurethane configured with therapeutic biomolecules or the respective unmodified control polyurethane. Polyurethane valve leaflets were implanted into the pulmonary position via a standard thoracotomy through the fourth intercostal space with the aid of cardiopulmonary bypass. At the conclusion of the study, sheep were euthanized with an overdose of barbituates and the valve was exposed, visualized, and removed for microscopic studies including immunohistochemical analysis.

2.3. Methods used for gene therapy arrhythmia studies

2.3.1. Molecular biology techniques

Bicistronic plasmid vectors expressing both the human minK-related protein-1 (h-MiRP1) sequence of interest (wild type) or the Q9E mutation of h-MiRP1 and GFP were prepared as published by our group [7]. These plasmids utilized the cytomegalovirus (CMV) promoter and also included both a FLAG sequence and a neomycin resistance element. Cell culture studies utilized either HEK 293 cells or SH-SY5Y neuroblastoma cells (7). Transfection was carried out with lipofectin 2000 and flow cytometry was used to sort GFP-positive cells [7].

2.3.2. In vitro methods

Single cell patch clamp studies were carried out under constant voltage conditions with cells transfected with either the wild type or Q9E plasmids [7]. Recordings were performed initially without clarithromycin (CL) and then with increasing amounts of CL. Inward potassium rectifier currents (IKr) currents were recorded and compared with and without CL administration.

2.3.3. In vivo methods

Male farm pigs (30 kg) were subjected under general anesthesia to a right thoracotomy and the right atrium was exposed [8]. Two epicardial sites in the right atrium, 2 cm apart were marked with a 4–0 vicryl suture and each site was injected with 100 μg of either the Q9E plasmid or in control sites either saline or wild type plasmid. After two weeks the animals were re-operated upon and monophasic action potential recordings (MAP) were obtained continuously over each site before and after a 20 mg/kg CL infusion was administered over a 30 min period (8). Surface electrocardiogram (EKG) tracings were recorded as well. At the end of the CL infusion the animals were euthanized and myocardial samples were analyzed histologically for the extent of gene expression.

3. Results and discussion

3.1. Gene delivery stents

3.1.1. Plasmid delivery from stents — bulk immobilization

Our laboratory was the first to demonstrate plasmid DNA delivery from a stent platform [1]. We examined PLGA stent coatings with DNA dispersed in the bulk of the PLGA.

Stent coating was achieved by multiple cycles of dipping stents into emulsion and evaporation to dryness. In a typical formulation a stent harbored 1 mg of DNA incorporated in ca 30 μm layer comprising 8–9 mg of PLGA. Using rhodamine-labeled DNA we documented a uniform distribution of plasmid DNA material throughout the coating, which was present both on the stent struts and between them in the form of a thin web-like film [1]. No fragmentation of PLGA was noted following forceful stent deployment. In vitro dissolution studies demonstrated a marked burst effect with 50% of the DNA load released after 1 h. The rest of the plasmid DNA was released with the first order kinetics over 10 days [1]. Released DNA retained an intact structure and was able to transfect cultured smooth muscle cells (SMC) when complexed with lipofectamine. The placement of DNA/PLGA-coated stent wires into SMC culture resulted in localized transfection of 7.9% cells proximal to the wires. Pig angioplasty studies with DNA/PLGA stents showed transfection rates of 1.14% of total cells in the stented segment with preferential transfection of the media, and to a lesser extent of neointima and adventitia [1]. Distal spread of vector DNA to the lungs was detected by PCR in 2 of 6 animals.

Later we refined the technique of stent-based plasmid delivery by the elaboration of heat-denatured collagen coating for plasmid DNA immobilization [2]. In addition to providing a matrix for DNA dispersion denatured collagen possesses the unique transfection enhancing mechanisms operating via $\alpha v\beta 3$ integrin receptor interactions and associated cytoskeletal actin modifications [2]. The deposition of carbodiimide cross-linked denatured collagen on the stent surface was achieved by repeated coating/drying cycles. In typical preparations, a total of 1 mg of collagen was admixed with 500 μg of DNA and was applied as a coating on a 15 mm Crown stent. For vascular implants a thin sealant layer of PLGA was formed on the surface of the collagen/DNA matrix since cell culture experiments have shown that the plasmid release rate from denatured collagen per se is too rapid with virtually all of the DNA load released in less than 30 min. On the other hand, the presence of the PLGA sealant prolonged DNA release beyond 24 h. In the pig coronary model GFP expression levels in the neointima 7 days after stenting were 9-fold higher for the collagen/DNA coated stents formulation than for the PLGA/DNA stents described above (10.4 ± 1.23%, vs. 1.14% ± 0.7%). Furthermore, denatured collagen-PLGA coated stents contained only half the amount of DNA that was formulated in the PLGA-DNA coated stents (500 vs. 1000 μg, respectively).

3.1.2. Surface immobilization of adenoviral vectors — the tethering strategy

The release of bulk immobilized plasmid DNA from the stent coating is driven by the combined effect of vector diffusion and matrix degradation and depends both on vector particles size and the chemical properties of matrix. Recently, our group described a novel approach for genetic vector surface immobilization based on the use of high affinity ligands to tether a vector to a substrate [9]. Surface tethering of genetic material bestows advantages of decreased vector dose and potentially better control of release kinetics compared to the bulk incorporation techniques. We pioneered affinity binding of adenoviral vectors by achieving successful surface immobilization of Ad in a collagen-avidin gel using biotinylated anti-hexon antibody for Ad coupling [9]. In rat arterial smooth muscle cell cultures this system was shown to enable site-specific localization of viruses encoding for the transgenes with reporter and therapeutic activities [9].

3.1.3. Ad tethering to stents using anti-knob antibodies

The SPDP-activated collagen films and collagen-coated stents (Fig. 1) were conjugated with anti-knob F(ab)' fragments allowing for affinity immobilization of recombinant Ad-GFP [3]. The loading capacity of the antiknob antibody-derivatized collagen films was determined by incubations with progressively increas-

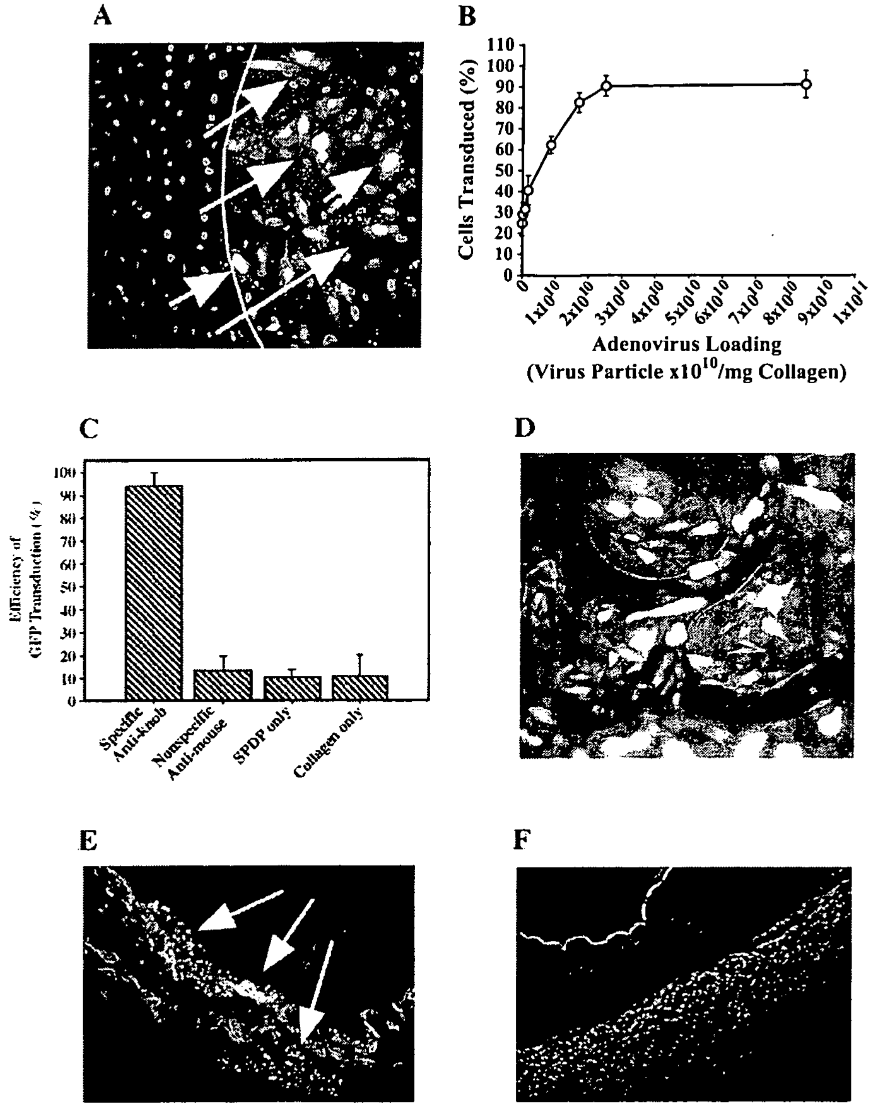

Fig. 1. Adenovirus delivery stent. (A) Strict spatial restriction of transduction in cell culture by Ad-GFP immobilized on the surface of a collagen film using an antiknob antibody as an affinity ligand. The rat arterial smooth muscle cells (A10) cells were DAPI-counterstained. The collagen film's border is delineated by white lines; the arrows point to transduced cells (Merged DAPI/FITC image, 200× magnification). (B) Viral loading determined by transduction efficiency following exposure of antiknob derivatized collagen films to successively higher levels of Ad-GFP demonstrates asymptotic transduction at 2.5×10^{10} viral particles/mg derivatized collagen. (C) Specificity of antibody affinity for vector tethering determined by the extent of transduction in cell culture. Properly derivatized collagen films containing antiknob $F(ab')_2$ are compared to control films containing either a nonspecific antibody, films reacted with SPDP cross-linker alone, or collagen films that were not exposed to the derivatization procedure ($p<0.001$ specific antibody tethering versus controls). (D) Antibody tethered Ad-GFP delivery from collagen-coated stents in A10 cell culture results in abundant spatially localized GFP expression (FITC, 200× magnification). (E) Pig coronary photomicrograph demonstrating arterial wall transduction following stenting with collagen-immobilized antiknob tethered adenovirus. Positive GFP expression (arrows) compared with (F) the control stent-treated artery demonstrating typical levels of autofluorescence (FITC, 400× magnification). Reproduced with permission from Klugherz et. al., [3].

ing Ad titers. The maximal amount of Ad associated with collagen-antibody formulation was shown to be 2.5×10^{10} particles (5×10^{8} PFU) per 1 mg of the film. A direct relationship was observed between viral loading and transduction efficiency (Fig. 1B). Incubation of the collagen-Ad films in physiologic buffer demonstrated high affinity retention of transduction-competent Ad in films with slow release of 30% of bound virus into the buffer over 20 days. In contrast, when Ad was passively associated with the collagen films without antibody as an affinity adapter, 80% of the Ad payload was released into the incubation buffer after 7 days. Adenovirus binding to films was highly specific. Transduction of 92.8 ± 2.5% of A10 cells ensued when virus was tethered by the anti-knob monoclonal antibody. In contrast, following addition of virus to collagen alone, SPDP-treated collagen, or collagen derivatized with nonspecific anti-mouse $F(ab')_2$ significantly lower efficiencies of transduction (13.2%, 10.2%, and 10.8%, respectively) were observed, reflecting the lower, nonspecific binding of adenovirus to these collagen matrices (Fig. 1C). In SMC culture both collagen-Ad films and collagen-coated Ad-tethered stents demonstrated intensive, site-specific transgene expression within 100 μm of the film or the stent wire borders (Fig. 1A and D).

To study in vivo transduction activity of stent-immobilized Ad, the Ad-GFP tethered stents were deployed into pig coronaries. Fluorescence microscopy analysis of the explants harvested 7 days after deployment revealed widespread GFP expression (Fig. 1E), which was only minimal in control segments treated with the stents conjugated with non-specific F(ab)' prior to incubation with Ad-GFP (Fig. 1F). GFP expression was confirmed by immunohistochemistry demonstrating a 17% transduction rate in neointima with a lower percentage of GFP-positive cells in media and adventitia (7% and 1%, respectively). The actual extent of GFP-positive cells in neointima was probably even higher, since a fraction of the transduced cells was found to be permanently attached to stents surface and thus lost during specimen evaluation. Since the neointima contributes to ISR this inward gradient of transduction is highly desirable, and probably is a result of direct physical contact of immobilized Ad with innermost elements of the vessel wall. A potential distal spread of Ad vector from delivery site was studied by PCR. A GFP signal was consistently found only in the arterial fragment underlying the stent, but not in non-stented arteries, myocardium, liver, kidney or lung [3].

The idea of using stents as gene delivery platforms poses a number of important challenges, both technical (preservation of mechanical properties, limited surface for immobilization, sustained release) and biological (inflammation, increased thrombogenicity, impaired endothelial in-growth). Nevertheless, since vector immobilization provides apparent therapeutic advantages by increasing effective local concentration and diminishing the burden of gene vectors on non-target organs, gene delivery stents were investigated over the last decade.

Takahashi et al. [10] reported successful arterial transfection in rabbit iliac arteries using a stent loaded with 96 μg of plasmid DNA immobilized within a layer of polyurethane formulated by a dipping technique. A conceptually different approach for stent-based gene therapy was suggested by Feldman et al. [11]. These authors used a microneedle-studded stent enabling focal delivery of plasmid DNA into rabbit arteries. Microneedles spaced 40–100 μm apart were loaded with 6.7 ± 0.6 μg DNA. The device was shown to be capable of effective transfection in cultured SMC, though no detailed in vivo study was carried out.

The first therapeutic study employing stent immobilized plasmid vector was recently undertaken with the plasmid encoding for VEGF [12]. The plasmid (100–200 μg per stent) was embedded in a phosphorylcholine coating. The VEGF plasmid-loaded stents achieved both higher endothelialization and functional recovery than the control stents, and resulted in smaller neointima and larger arterial lumen 4 weeks after deployment in hypercholesterolemic rabbits.

Several other groups [13–15] have investigated focal arterial transduction using polymer-coated stents to deliver bulk-immobilized Ad vectors and were able to show prolonged reporter activity in organ culture and in vivo. Moreover, a therapeutic effect of phosphorylcholine stent coating-immobilized adenovirus encoding tissue inhibitor of metalloproteinase-3 on neointimal formation was recently demonstrated in the pig model [16].

Thus our research, as well as studies from other laboratories has firmly established feasibility, safety, and in some instances therapeutic activity of gene delivery stents as anti-restenotic devices. However, much work has to be done before gene delivery stents can advance toward clinical use. Presently the most important task is the curtailing of coating-related chronic inflammation, which is responsible for the lost of initial therapeutic gain in some studies examining long-term effectiveness of the drug eluting stents. To this end, recent discoveries in our laboratory have led to investigations of bisphosphonate coordination chemistry for attaching viral gene vectors via antibodies or receptor-peptides to bare metal stents [17]. This surface-oriented approach virtually eliminates the need for a stent coating. Our preliminary studies have not revealed any increased inflammatory reaction towards poly-bisphosphonate modified versus bare metal stents. Furthermore, affinity attachment of adenoviral vectors to the polybisphosphonate modified stents has demonstrated successful gene transfer in the rat carotid model, and a therapeutic effect with an Ad vector driving the synthesis of the inducible isoform of nitric oxide synthase.

3.2. Gene therapy for heart valve disease

Heart valve disease represents the second most frequent indication for open-heart surgery, behind coronary artery disease. Nevertheless, there are no medications for heart valve disease, and the only therapeutic approach at this time is heart valve replacement, or attempts at surgical reconstruction of diseased heart valve tissue. Investigations in our laboratory have demonstrated that polyurethane prosthetic heart valve leaflets can be configured with immobilized adenoviral gene vectors that can successfully modify interacting cells. Furthermore, recent studies from our laboratory have demonstrated that genetic engineering of blood outgrowth endothelial cells can be used to provide a cell therapy approach to gene delivery for heart valve disease by seeding cholesterol modified polyurethane with genetically engineered blood outgrowth endothelial cells. Thus, site-specific modification of prosthetic heart valve leaflets may represent a powerful approach for gene delivery to treat heart valve disease.

Polyurethane elastomers have favorable physical properties, such as durability and elasticity, which make them an attractive material for the construction of prosthetic heart valves. However, in vivo and clinical trials of polyurethane valve implants noted thrombi formation and ectopic calcification on the prosthetic valve surface [18,19]. Addressing these pathologies by appending therapeutic moieties to the polyurethane was confounded by the elastomer's lack of reactive chemical groups. We recently developed and characterized a chemical synthesis whereby hard segment nitrogens are bromoalkylated and subsequently reacted with thiolated moieties. Initial studies using this bromoalkylation strategy addressed ectopic calcification of polyurethanes by covalently appending the anti-calcification moiety bisphosphonate to a polycarbonate polyurethane (Biospan). These studies showed extended polyurethane resistance to calcification in a rat subdermal implant model as well as a sheep circulatory implant for 60 and 150 days, respectively, without any adverse effects on growth [20].

Subsequent results from our laboratory have used bromoalkylated polyurethane as a platform for gene delivery from vascular implants (Fig. 2). The surface of the polyether polyurethane (Tecothane® TT1074A) was configured with covalently attached anti-adenovirus antibodies. Replication defective adenoviral vectors containing within their genome the gene encoding green fluorescent protein (Ad-GFP) was tethered to the polyurethane via antibody-epitope recognition. The virus-loaded polyurethane was fashioned into heart valve leaflets and replaced a single pulmonary valve leaflet in a juvenile sheep. In vivo results showed localized adenovirus mediated gene delivery from 25% of cells attaching to the surface of the polyurethane valve as well as 18% of cells in the adjacent myocardial tissue. Of importance, PCR analysis showed no GFP in untargeted tissue such as lung, liver, blood, and spleen. These results strongly suggested that gene delivery was localized only to the region of the replacement valve. Immunohistochemical analysis showed the cells seeding on the valve to be almost exclusively leukocytes [4].

To address thrombi formation on the valve surface we have investigated strategies of enhancing endothelial cell retention on polyurethane under physiological conditions. Recently we have developed and characterized polyurethane configured with covalently bound cholesterol moieties [5]. Our choice of cholesterol was based on intrinsic properties of the

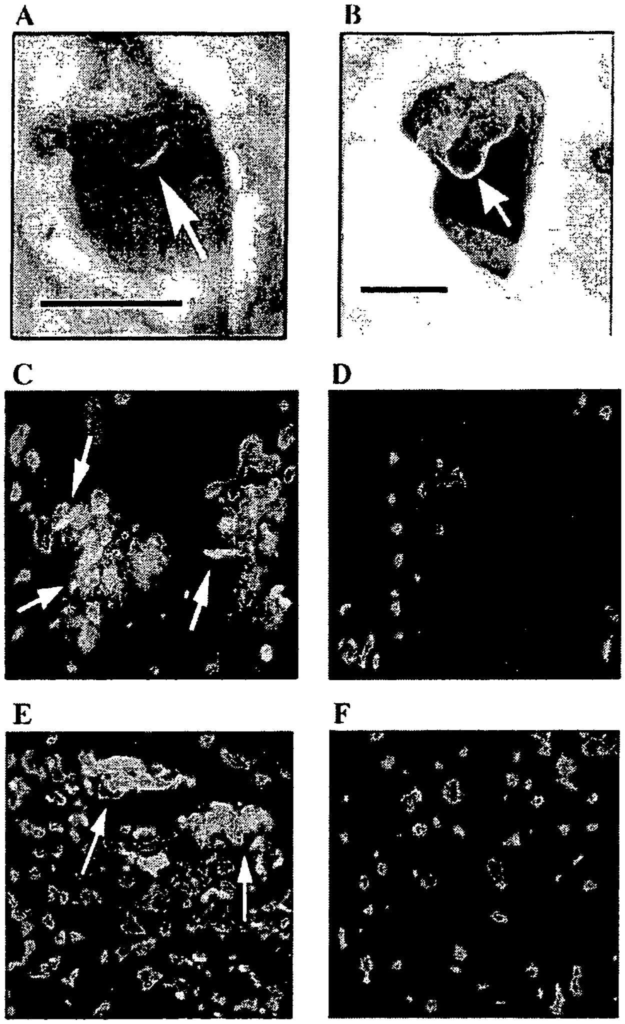

Fig. 2. Heart valve leaflet gene delivery using polyurethane (PU) pulmonary replacement cusps in sheep with antibody tethered Ad-GFP (10^8 PFU). (A) The gross appearance (pulmonary outflow side shown) of an Ad-GFP-polyurethane sheep pulmonary leaflet explant demonstrating a smooth blood contacting surface. (B) A non-modified polyurethane pulmonary leaflet replacement at explant (also shown from the pulmonary outflow side), demonstrating a smooth blood contacting surface, comparable to (A). (C through F) GFP expression demonstrated by fluorescent microscopy (FITC) with DAPI counterstaining in representative explanted polyurethane pulmonary cusp replacement (as in A, above) with cell localized GFP (C) expression on the surface of the Ad-GFP appended cusp (see arrows) 400×. (D) Absence of autofluorescence in a control (no adenovirus vector) polyurethane cusp explant per fluorescent microscopy (FITC) with DAPI counterstaining (as shown in B, above) 400×. (E) GFP expression in sheep myocardium adjacent to the Ad-GFP-polyurethane cusp implant (as in C, above). FITC/DAPI, 400×. (F) An absence of FITC autofluorescence in myocardium adjacent to control (no adenoviral vector) polyurethane cusp implants. FITC/DAPI, 400×. Reprinted by permission from Stachelek et. al., [4].

cholesterol molecule that were hypothesized to be of importance in this setting. Cholesterol's steroid ring offers an element of degradation resistance that other biomolecules such as phospholipids cannot provide. There is also growing evidence showing cholesterol to be a necessary component of cell surface mediated signaling pathways [21,22] and adhesion. Shear flow experiments carried out by our group [5] with bovine aortic endothelial cells (BAECs) and blood outgrowth endothelial cells (BOECs) seeded on polyurethane ± covalently appended cholesterol showed that cholesterol modified polyurethane had superior cell retention properties when either endothelial cell type was exposed to arterial (25 dynes/cm^2) or valvular levels of shear (75 dynes/cm^2).

BOEC seeded polyurethane represents an important strategy for cell-based therapies. Recently retrovirus transduced human BOECs expressing human Factor VIII were injected in a NOD/SCID mouse and therapeutic levels (10 ng/ml) of Factor VIII were detected after 150 days [23]. In our laboratory we note that BOECs are readily transduced with lentivirus (Fig. 3) with complete transduction at a multiplicity of infection at 250 PFU/cell. Future therapeutic strategies for heart valve replacements can involve lentivirus transduced autologous BOECs seeding of prosthetic heart valves composed of cholesterol-modified polyurethane. Candidate genes, such as tissue plasminogen activator (TPA) or hirudin, secreted from seeded autologous lentiviral transduced BOECs would hypothetically serve as localized cell mediated gene delivery platform to alleviate thrombi formation on the prosthetic valve. The combination of genetically modified BOECs seeded on a high affinity surface, such as cholesterol modified polyurethane, is a promising strategy for cell based gene delivery from an implantable vascular device.

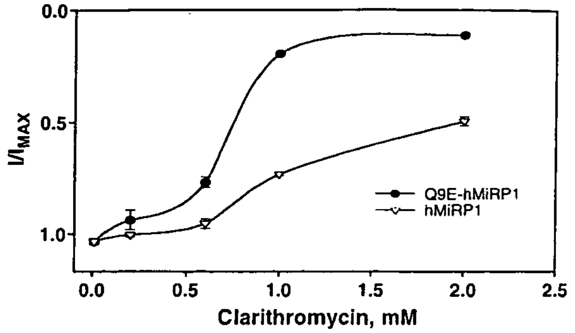

Fig. 4. The results of single cell voltage clamp studies using HEK293 cells with stable plasmid transfection of either wild type hMiRP1 or the mutation, Q9E-hMiRP1 demonstrating a clarithromycin-dependent reduction in normalized potassium inward rectifier currents (I/IMAX). Reproduced with permission of the publisher from Burton et al, [7].

3.3. Gene therapy for cardiac arrhythmias

Cardiac arrhythmias are the most frequent cause of sudden death, and affect millions of patients on a chronic basis. Nevertheless, all of the pharmaceuticals

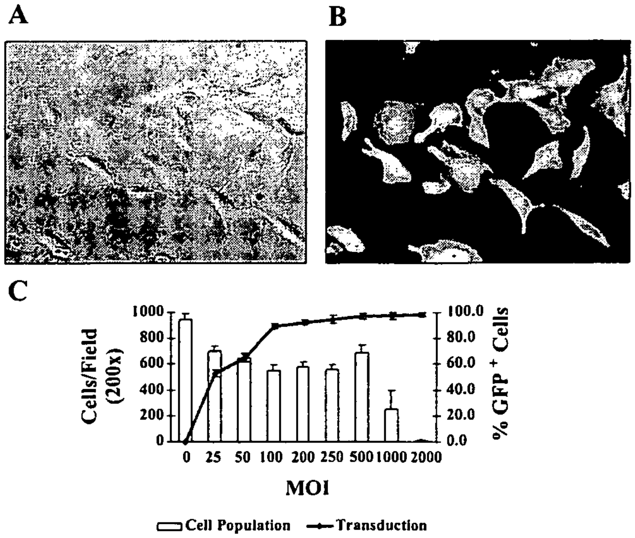

Fig. 3. Transduction efficiency of lentivirus mediated gene transfer in cultured BOECs. Phase micrographs (A) and corresponding fluorescent (FITC) image (B) of BOECs transduced with ^{GFP}Lentivirus at a multiplicity of infection (MOI) of 250 PFU. All cells in field were positive for GFP expression. Images were acquired at 200× (C) Graphical representation of BOEC cell number (Bar) and GFP expression (Line) at increasing MOI. Maximal transduction efficiency was attained at an MOI of 200.

used for cardiac arrhythmias are incompletely effective, and demonstrate major adverse effects. Thus, a gene therapy approach was investigated for treating this important class of disorders. An ion channel mutation (Q9E-hMiRP1) [7,24] was studied because of its unique responsiveness to the macrolide antibiotic, clarithromycin (CL). Q9E, a mutation of the potassium inward rectifier ion channel, hMiRP1, was configured in a plasmid vector, and investigated both in patch clamp studies and in vivo [7,8]. CL responsiveness was demonstrated both in vitro and in vivo. Single cell patch clamp studies show the dose dependence of the IKr currents with increasing amount of CL (Fig. 4) in cells transfected with Q9E plasmid DNA. Cells transfected with wild type DNA demonstrated significantly less suppression of IKr with CL.

In vivo studies in pigs [8] demonstrated that with CL administration Q9E transfected sites had site specific prolongation of the epicardial monophasic action potential duration (MAP) whereas sites transfected with a wild type (hMiRP1) plasmid did not demonstrate CL responsiveness (Fig. 5), and thus showed no change in MAP duration. The localized electrophysiologic effects demonstrated are comparable to those achieved with Class III anti-arrhythmic drugs, the agents used most widely to treat atrial arrhythmias [24]. There were no cardiac arrhythmias associated with CL administration, and the surface EKG was also unaffected by CL administered in these studies. Importantly, no prolongation of the QT interval was noted, indicating the highly localized interaction of CL with the Q9E transgene [7].

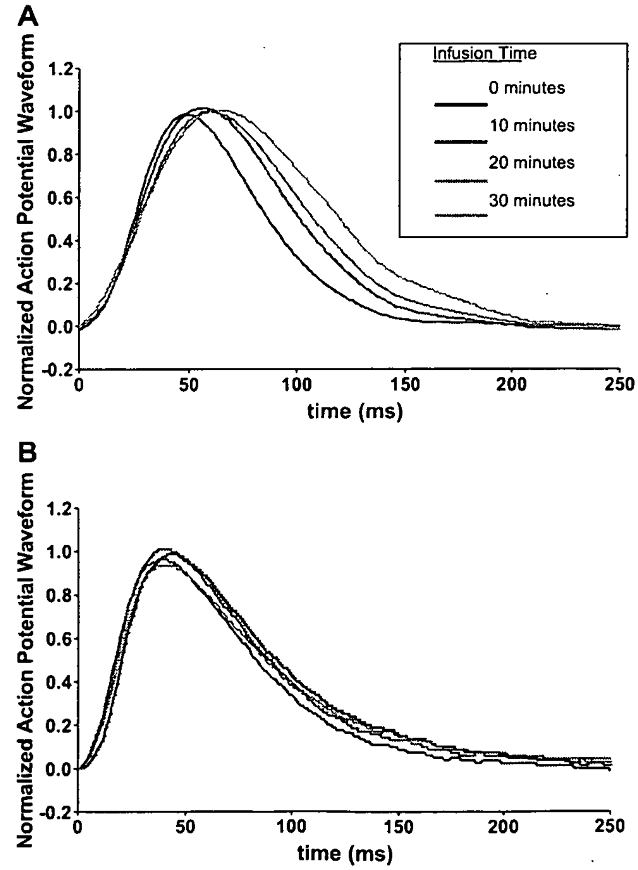

Fig. 5. In vivo site specific reduction of the repolarization phase of the atrial epicardial monophasic action potential in pigs only over the Q9E-hMiRP1 transfected site (A) compared to a control site transfected with wild type plasmid (hMiRP1) 2 cm distal (B) showing no change in MAP duration despite a clarithromycin infusion. Reproduced with permission of the publisher from Perlstein et al, [8].

The gene therapy strategy just described for treating cardiac arrhythmias has a number of important features. It represents the first gene therapy approach for any disorder that utilizes a post translational control mechanism thus permitting regulation of transgene activity with a pharmaceutical agent that does not have any pharmacological activity for the disease process of interest. Of additional importance, the transgene that was used, Q9E, is a disease associated mutation that is responsible for one of the drug associated forms of the long QT syndrome [24], a hereditary rhythm disorder that can result in ventricular arrhythmias and sudden death. However, the present studies used only atrial administration of the gene vectors encoding this mutation, thus avoiding ventricular exposure completely. Hence, no ventricular arrhythmias were noted. Furthermore, site specific administration of the vector resulted in highly localized transgene activity and this means of administration should also hypothetically result in treatment of a tightly defined region of interest. Thus, this approach will optimize local efficacy and minimize of adverse affects.

One of the major drawbacks of the just described approach is the delivery method involving atrial myocardial injections. The thinness of the atrial myocardium and the needle track patterns of gene administration make this approach suboptimal. Investigations by others [25] have used epicardial hydrogels with enzymatic digestion of the myocardial extracellular matrix. Although this is an innovative means of delivery this route of administration is not practical for clinical use. Targeted ferric oxide nanoparticle delivery of vectors with localization based on magnetic gradients may offer the greatest promise [26]. Cell based therapies utilizing genetically engineered stem cells containing transgenes such as Q9E could be an ideal approach. However, cellular delivery is even more challenging than gene vector delivery, since a number of aspects of cell based therapies are not as yet well understood. For instance, at present integration of stem cells into the myocardium is not controllable, either from an electrophysiologic or biomechanical point of view. In addition myocardial cell based therapies have been frequently pro-arrhythmic in both experimental and clinical studies. Nevertheless, tissue engineering constructs with scaffold-based organization of myocardial precursors may offer an innovative strategy for a cell based anti-arrhythmia treatment.

4. Conclusions

Site-specific gene delivery in the cardiovascular system has been demonstrated to be both feasible and efficacious in experimental studies. The gene delivery techniques involved can be adapted for use in a variety of cardiovascular diseases including arteriosclerosis, heart valve disease, and cardiac arrhythmias.

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A concise peer into the background, initial thoughts and practices of human gene therapy

Manuel A.F.V. Gonçalves

Summary
The concept of human gene therapy came on the heels of fundamental discoveries on the nature and working of the gene. However, realistic prospects to correct the underlying cause of recessive genetic disorders through the transfer of wild-type alleles of defective genes had to wait for the arrival of recombinant DNA technology. These techniques permitted the isolation and insertion of genes into the first recombinant delivery systems. The realization that viruses are natural gene carriers provided inspiration for gene therapy and, as engineered vectors, viruses became prominent gene delivery vehicles. Nonetheless, when put in the context of human and non-human primate studies, all vectors fell short of success regardless of their viral or non-viral origin. Recognition of issues such as inefficient gene transfer and short-lived or scant expression in the relevant cell type(s) prompted researchers to refine and develop several gene delivery systems, in particular those based on retroviruses, adeno-associated viruses and adenoviruses. Concomitantly, available technology was deployed to tackle disorders that require few genetically corrected cells to attain therapy. *BioEssays* 27:506–517, 2005.
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Introduction

Gene therapy can be defined as the delivery and expression of genetic information in cells of an individual to restore health or to ease the consequences of disease. Initially designed for monogenetic recessive disorders, gene transfer technologies, partially owing to the early failures in achieving long-term therapeutic gene expression,(1) have also been adapted to tackle complex traits such as certain cardiovascular defects(2) and cancer(3) as well as infectious diseases (Fig. 1).(4) Recently, allelic mutations associated with dominant congenital disorders are also being targeted at the RNA(5,6) and DNA(7) levels by gene transfer approaches (Fig. 1). Strategies targeting inherited and acquired diseases, including viral infections, at the RNA level received an enormous boost with the demonstration that, in mammalian cells, small double-stranded RNA can induce degradation of transcripts containing a complementary sequence. These small interfering RNA (siRNA) molecules are proving highly versatile tools for gene knockdown studies. The therapeutic use of RNA interference will obviously require additional work to overcome, amongst others, the hurdles of efficient siRNA delivery and off-target activity.(8)

While disarmingly simple as a concept, in practice human gene therapy has not yet delivered its full potential, which was initially thought to be attainable with relative ease by exploiting the flow of information arising from the study of life at the molecular level.(9) The use of this flow of information has here a literal meaning since at the core of this "new biology" were the stepwise discoveries collectively known as "the central dogma of molecular biology", proclaiming that genetic information stored in DNA is channelled via mRNA molecules into the function-bearing proteins. Hence, altered genes often translate into non-functional gene products leading to disease. As a corollary, transfer of wild-type alleles of crippled genes should complement the phenotype of recessive disorders. Thus, the work of pioneering molecular biologists had a crucial impact on the development of gene therapy at two levels. Firstly, it provided a theoretical framework for action by demonstrating and dissecting the genetic basis of human pathology and, secondly, it generated the methods and tools for gene transfer itself. Nowadays, these fundamental and technological developments run in parallel and are being integrated as seen by the rapidly growing list of mutations associated with specific disease traits and the design of increasingly sophisticated gene delivery systems (vectors). However, when the idea of human gene therapy was advanced, independently, by forward-looking investigators, there was a gap in the technical part of the endeavour. This gap started to be filled when recombinant DNA technology and methods to introduce nucleic acids into cells and to select for genetically modified cells became available.

The present review aims at providing an overview of the main original ideas and experiments that were the basis that helped to shape human gene therapy. Simultaneously, the basic principles that are at the core of this rapidly evolving and

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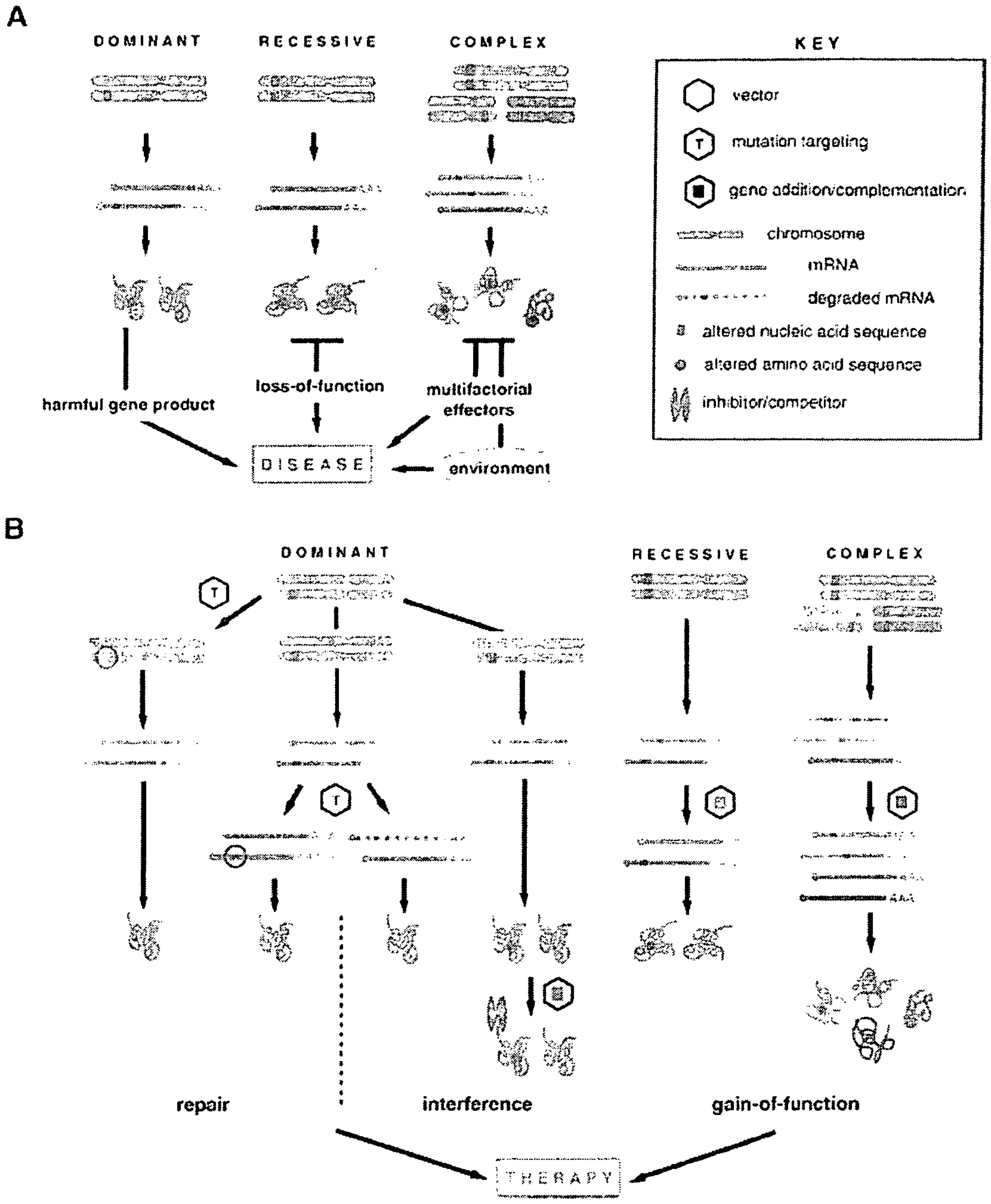

Figure 1. **A:** Generic causes of disease. Dominant monogenetic disorders (e.g. Huntington) are caused by a mutated allele that translates in a harmful gene product. Ultimately, this product can interfere with the normal physiology of the cell/organism. Deleterious gain-of-functions can also be acquired (not drawn), for example through viral infection (e.g. acquired immunodeficiency syndrome). Recessive monogenetic diseases (e.g. cystic fibrosis) are caused by mutations in both alleles of an autosomal gene or, in males, in the single allelic copy of an X-linked gene (e.g. Duchenne muscular dystrophy and hemophilia B). This leads to the synthesis of a non-functional gene product required for the normal physiology of the cell/organism. Complex disease traits are caused by the interplay between several gene products. This class of ailments can also have a non-hereditary, environmental component (e.g. cardiovascular defects and cancer). **B:** Possible therapeutic interventions at the genetic basis of disease. Approaches aimed at tackling dominant monogenetic disorders have to repair or interfere with the altered genetic information either at the DNA (e.g. via targeted homologous recombination), RNA (e.g. via exon skipping and RNA interference) or protein (e.g. via *trans*-dominant negative effects) levels. Efforts to cure recessive monogenetic and multi-gene disorders normally rely on gene addition strategies that simply complement the defective genotype or provide cells with a new function. Although, frequently, the etiology of multi-gene disorders is not precisely known, gene transfer procedures can endow defective cells with a new function that overcomes the disease phenotype (e.g. cardiovascular defects) or triggers target cell death (e.g. cancer).

highly interdisciplinary branch of biomedical research (e.g. transduction, transfection, ex vivo and in vivo gene therapy, vector design) are discussed in their historical context.

Gene transfer before recombinant DNA technology

In 1952, it was established that a so-called "filtrable agent" identified as a virus of bacteria (bacteriophage) could transfer discrete genetic markers (e.g. nutritional requirements and drug resistance) to *Salmonella*.(10) In the same study, the term transduction was introduced as follows: "The mechanism of genetic exchange found in these experiments differs from sexual recombination in *Escherichia coli* (*E. coli*) in many respects so as to warrant a new descriptive term, transduction". Also in the early 1950s, the idea of prophage as bacteriophage genomes inserted in the DNA of host bacteria emerged as part of an explanation for the puzzling phenomenon of lysogeny.(11) This concept of prophage, later experimentally vindicated and dissected in its finest molecular detail,(12) was extended to eukaryotic viruses through the provirus hypothesis of Howard Temin. This hypothesis was based on results showing that chicken cells infected with the Rous sarcoma virus (RSV) stably inherited both morphological changes associated with specific viral gene mutations and information for the generation of RSV progeny.(13) Interestingly, this hypothesis gave rise to a conundrum since it was known at the time that the RSV genome consists of RNA,(14) whereas the molecular nature of the provirus is by definition DNA. Thus, the provirus hypothesis challenged the abovementioned "central dogma of molecular biology" by postulating that genetic information can flow not only downstream from DNA to RNA but also upstream from RNA to DNA. The subsequent discovery of RNA-dependent DNA polymerases(15,16) together with experiments demonstrating hybridisation of labelled RSV RNA to DNA of infected chicken cells(17) established the provirus concept. Moreover, studies contemporary with those using RSV based on nucleic acid fractionation and hybridisation techniques, were performed on cells transformed by the polyoma virus and simian virus 40 (SV40).(18) From these experiments, it also emerged that the genomes of these DNA viruses were found integrated in the chromosomal DNA of the transformed cells. Thus, at this time, the notion that viruses can be regarded as packages of nucleic acids that can introduce (transduce) new activities or processes into host cells, such as cellular transformation, was well established. Furthermore, it was also realized that these newly acquired characteristics could be stably inherited by daughter cells through chromosomal insertion of the foreign genetic material.

Hence, before the advent of recombinant DNA technology, it was already clear that viruses evolved a set of attributes that could turn them into ideal gene delivery vehicles. Chief among these was the efficiency with which they can stably transduce their genomes into host cells. The challenge presented to those involved in the development of viral vectors would be to strip these natural gene carriers from the genetic payload involved in host cell hijacking or tumour formation and graft therapeutic genes instead. For instance, in 1966, Tatum and Rogers anticipated independently the use of viruses as vectors to transfer isolated or newly synthesized genes into defective cells.(19,20) Thus, in some sense, the simplified definition of Medawar of a virus as "a piece of bad news wrapped up in protein"(21) should be modified by exchanging the adjective for its antonym to define a viral vector as "a piece of good news wrapped up in protein".

Despite the aforementioned technical barriers an initial proof of concept for the transfer of foreign genetic information by a designed virus (viral vector) was demonstrated in 1968 in an article titled "Use of viruses as carriers of added genetic information".(22) In this study, the investigators used polynucleotide phosphorylase to chemically modify the infectious genome of the tobacco mosaic virus (TMV) by the addition of a polyadenylate [poly(A)] stretch to purified TMV RNA. Cellular lysates from tobacco leaves infected with the modified TMV viruses had elevated levels of tetra- and penta-lysine oligomers indicating the expression of the genetic information added in the form of a poly(A) sequence. The authors concluded that the next step would be "to build a meaningful sequence on a virus RNA or DNA, replicate the modified virus to the amount needed, and use the virus to transmit the information". However, the technology "to build a meaningful sequence" was not available and their work on vector development ceased. Instead, backed by their expertise on the Shope papilloma virus (SPV) and on circumstantial evidence pointing to the existence of an SPV-encoded arginase activity,(23) Rogers and colleagues went on to perform the first direct human gene transfer study for an inherited disease using the wild-type SPV to treat two arginase-deficient girls. In addition to the incomplete understanding of hyperargininemia pathogenesis, this human gene transfer experiment was hasty since other studies did not support the presence of an arginase gene in the SPV genome.(24,25) The outcome was no change in the arginine levels or in the clinical course of the hyperargininemias. Later, the complete genomic sequence of SPV settled that the virus does not encode an arginase.(26)

Before recombinant DNA techniques emerged, the wild-type herpes simplex virus (HSV), known to encode a thymidine kinase (TK) activity, was used to convert Ltk$^-$ cells from the TK-negative to the TK-positive phenotype. The investigators applied UV-irradiated, nonlytic, viral particles to prevent target cell death. The cells that acquired the viral *tk* gene were selected by letting them grow in HAT medium containing hypoxanthine, aminopterin and thymidine. After these manoeuvres, the authors summarized their data as follows: "To our knowledge, this is the first instance in which a gene of a known function has been stably acquired by an eukariotic cell line due to virus infection. This may be a first step in establishing a system in which viruses are used as carrier mechanisms to enable desired information to be transferred to a mammalian cell and to be established in an inheritable fashion".(27)

Gene transfer after recombinant DNA technology

Since the mid 1960s, it was known that linear bacteriophage λ genomes could be circularised in vitro due to the presence of complementary cohesive ends at their termini.(28) This finding inspired the in vitro assembly of recombinant DNA following enzymatic generation of compatible cohesive ends at the extremities of disparate DNA molecules. After base-pair hybridisation through hydrogen bonding, a DNA ligase treatment would ensure the covalent sealing of the union. Reports on the construction of recombinant DNA molecules by these kinds of techniques first appeared in 1972 and 1973 heralding the arrival of recombinant DNA technology.(29) Despite an initial moratorium self-imposed by the scientific community to reflect on the potential dangers of the new technology,(30) complex libraries of cloned DNA segments containing genes and gene clusters from different species were available by the end of the 1970s. Essential to the latter and subsequent developments, such as DNA sequencing, was the demonstration by Smith and Wilcox of the existence of sequence-specific DNA cutters after their isolation of the first class II restriction endonuclease.(31) Equally important was a study by Danna and Nathans showing the remarkable power of combining sequence-specific phosphodiester bond cleavage with gel electrophoresis techniques to dissect large DNA molecules into discrete and manageable fragments.(32) As with virtually all technological breakthroughs, the discovery of restriction enzymes has its roots in fundamental research. Particularly, on observations from the early 1950s that bacteriophages propagated in one strain of bacteria grew poorly or were "restricted" in a second strain. A few viruses, however, escaped restriction and multiplied efficiently in the new strain.(33) The first step towards the elucidation of these phenomena was the discovery that restriction and resistance-to-restriction were associated with destruction and host-specific modification of bacteriophage DNA, respectively. Ultimately, endonucleases and methylases with common recognition sequences were identified as the culprits behind DNA restriction and modification/protection, as first postulated by Arber.(34)

Recombinant molecules containing SV40 DNA were among the first to be made. Interestingly, such hybrid molecules were built to create recombinant SV40 viruses to deliver non-viral DNA into cultured mammalian cells to, ultimately, study the function and regulation of eukaryotic genes. Thus, it was envisaged that this approach would emulate the successes that bacteriophage-mediated gene transfer had had on the analysis of bacterial genomes. The suggestion that such vectors could be made was based on results demonstrating that SV40 deletion mutants could be propagated using suitably matched SV40 temperature-sensitive mutants as helpers.(35) Following this study, Goff and Berg substituted segments of the SV40 genome for exogenous DNA in vitro. Applying the helper-dependent complementation principle to these defective SV40 genomes, they rescued the first recombinant viral vectors carrying, initially, bacteriophage λ sequences(36) and later an *E. coli* and a *Saccharomyces cerevisae* gene.(37) Similarly, others co-pioneered the construction and propagation of recombinant SV40 viruses containing either a λ DNA fragment(38) or an *E. coli* suppressor tRNA gene.(39) Importantly, in their experimental design, the investigators retained the viral origin of replication (ori) and kept the size of the hybrid molecules below 5.3 kilobases (the circular double-stranded SV40 DNA has 5243 nucleotide pairs) to enable recombinant DNA replication and incorporation into viral particles, respectively. These initial vectors did not lead to the synthesis of discrete transgene-specific mRNA and protein molecules. The absence of RNA splicing was later identified as the culprit for the lack of expression of functional products. The first successes in accomplishing transduction and proper expression of added genetic elements employed SV40 vectors carrying the rabbit *β-globin* full-length cDNA,(40,41) synthesized in vitro from mRNA using reverse transcriptase and DNA polymerase I,(42) the genomic mouse *β-globin*(43) or *α-globin*(44) genes or the bacterial *Ecogpt* gene.(45) These early vectors relied on template amplification in cells permissive for SV40 replication to induce high levels of heterologous protein synthesis. This approach had, however, limited utility since it invariably led to the death of the host cells precluding long-term genetic complementation studies. In addition, the reputation of SV40 as a transforming virus and, to some extent, its limited packaging capacity discouraged its general use as a gene therapy vector. Nevertheless, these recombinant SV40 experiments laid down some of the basic principles to be recapitulated in future vector development strategies (Fig. 2), that is substitution of *trans*-acting viral sequences by foreign DNA, linkage of the foreign DNA to *cis*-acting viral elements (e.g. origins of replication) and complementation of the deleted *trans*-acting viral sequences by segregated helper templates.

The introduction of molecular cloning during the early 1970s eventually provided methods not only to build recombinant virus genomes but also to isolate and clone mutant and wild-type alleles of disease-associated genes. These reagents were incorporated naturally in the protocols developed to introduce naked nucleic acid molecules into (transfect) mammalian cells in culture and to select, expand and characterize those that acquired the exogenous DNA.(46,47) Of note is the introduction in 1973, by Graham and van der Eb, of an improved calcium phosphate transfection technique.(48) Although previous work had implicated calcium phosphate precipitates in the increased uptake of nucleic acids by cells in culture,(47) in their study, Graham and van der Eb evaluated systematically the influence of a broad panel of experimental parameters on transfection efficiencies. It was determined that precipitates of DNA, calcium and phosphate were required for efficient transfection. This detailed study made the calcium-phosphate-mediated DNA transfection technique widely accepted, mainly due to its reproducible, 2-log higher efficiency over the, then more common, diethylaminoethyl-dextran method.(49)

Applying a modified calcium phosphate transfection protocol together with DNA from methotrexate-resistant dihydrofolate reductase (DHFR)-positive cells, Cline and colleagues showed induction of resistance to methotrexate in cultured mouse bone marrow (BM) cells. The transfected cells were subsequently transplanted into recipient mice conditioned to receive the cell graft by X-ray irradiation. After transplantation and methotrexate administration, the authors reported the presence of BM cells with the donor karyotype and higher DHFR enzymatic activity.(50) A contemporary study from the same laboratory followed similar gene transfer and transplantation regimens but used instead recombinant DNA molecules encoding as marker the HSV TK.(51) Based on these initial results, the researchers rapidly deployed the calcium phosphate transfection method to tackle human β-thalassemia. BM cells from two patients were harvested, transfected with the human *β-globin* gene outside the body (ex vivo) and

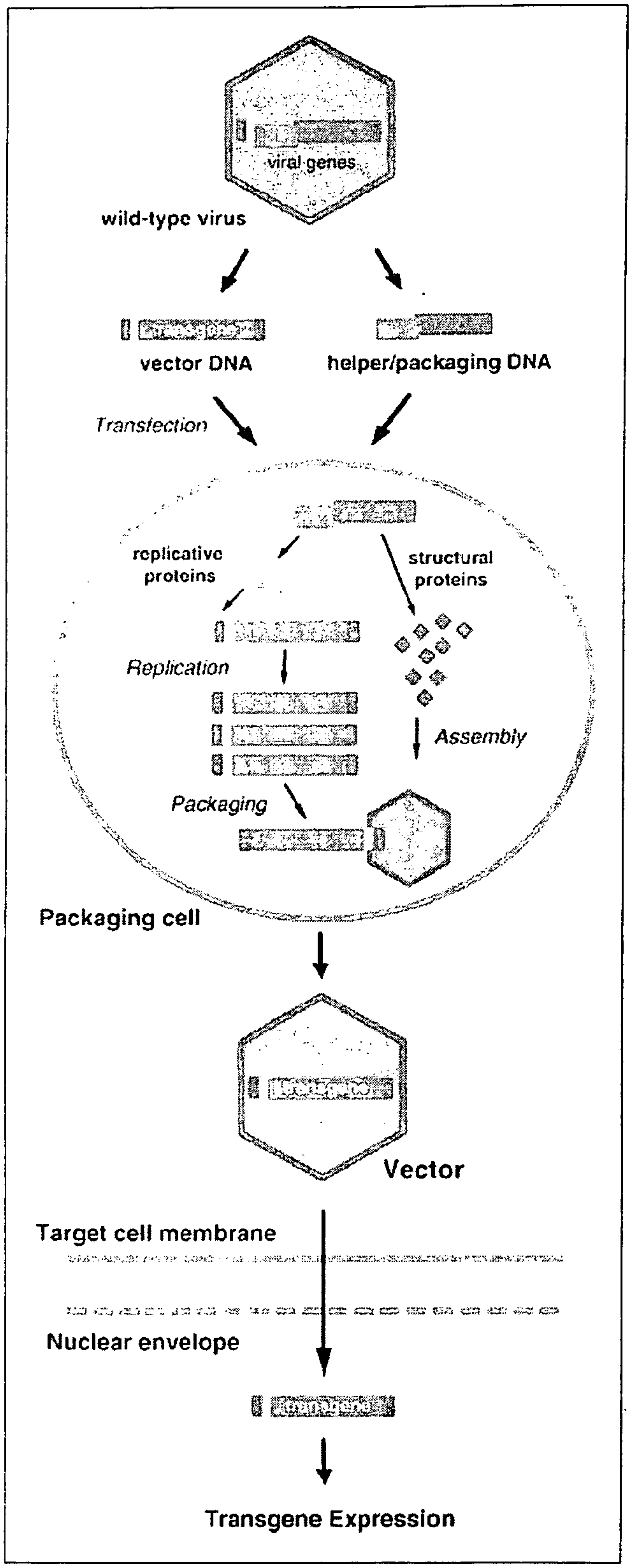

Figure 2. From virus to vector. The generic strategy to engineer a replication-competent virus into a replication-defective vector consists of segregating *cis*-acting sequences, namely, replication (red) and packaging elements (yellow) from *trans*-acting sequences that encode the viral non-structural and structural proteins (light and dark green, respectively). Removal of viral-coding sequences also generates space for a transgene expression cassette (cyan) consisting of promoter, transgene and transcriptional terminator sequences. Vector particles carrying the expression cassette are assembled by transfecting vector DNA into cells expressing helper functions (packaging cells). These functions can be provided in established packaging cell lines or be introduced into vector-producer cells by helper virus infection or DNA transfection. The replicative proteins (green triangles) recognize the viral replication sequences leading to the amplification of the vector genome (RNA or DNA depending on the type of vector) whereas the structural proteins (green squares) assemble into viral particles. Finally, the presence of packaging elements in the vector templates selects them for encapsidation from the complex nucleic acid mixture of the cell, originating infectious vector particles. Importantly, the absence of packaging elements in the helper constructs prevents their incorporation in viral particles. The vector particles are replication-defective and therefore can only undergo the first stages of the parental virus life cycle. This abortive infection of the target cell consists of cell entry and delivery of the transgene expression cassette into the nucleus without subsequent viral replication and spread.

transplanted back into the respective individuals. Breach of procedures necessary for approval led the host university and the National Institutes of Health (USA) to impose penalties on the researchers and to halt this first attempt at ex vivo human gene therapy. At the time, some scientists voiced their reservations over this approach. With hindsight and with the current knowledge on the inefficiency and transient nature of gene transfer provided by DNA transfection on the kind of BM cells required for haematopoietic cell reconstitution i.e. haematopoietic stem cells (HSC), the unsuitability of this method becomes evident.

HSC are very rare cells (0.001% to 0.01% of BM mononuclear cells). Therefore, any method to stably transduce these cells must, firstly, be very efficient and, secondly, must ensure the maintenance of the exogenous genetic material. Furthermore HSC are usually quiescent, although to maintain the homeostasis of the haematopoietic compartment they have the capacity to self-renew and, after a cascade of cellular divisions and differentiation stages, originate all the mature haematopoietic cell types (Fig. 3). These two defining functional properties of a stem cell, self-renewal and pluripotentiality, turn them into highly attractive targets for gene therapy, since stable genetic correction at the stem cell level would in principle ensure dissemination of the therapeutic DNA throughout the haematopoietic system.

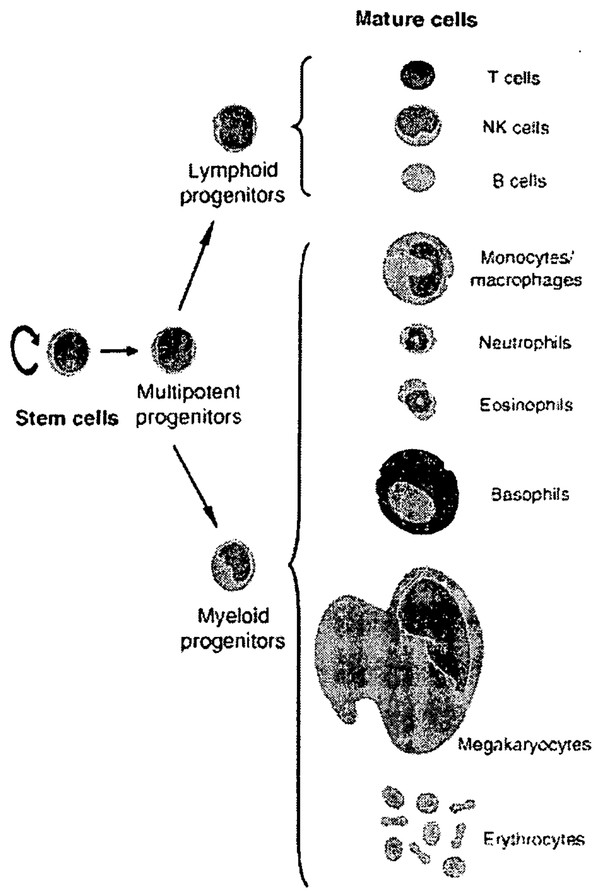

Figure 3. Illustration of key cellular entities of the haematopoietic system and their relative position in the developmental hierarchy. Long-living stem cells lie at the apex of the developmental hierarchy. These normally quiescent cells support and regulate haematopoiesis by dividing without differentiation (self-renewal) and by dividing and differentiating into multipotent progenitors with limited self-renewal potential. Further differentiation generates single-lineage progenitors (lymphoid and myeloid) that, via ever more developmentally restricted maturing precursor cells (not drawn), originate the mature function-bearing cells that constitute each of the different haematopoetic lineages.

Retroviral gene transfer and beyond

Prospects to stably transduce elusive HSC got a boost in the early 1980s with the introduction of the first retrovirus-based vectors.(52–54) These vectors retain desirable features of their parental viruses such as relatively efficient cell entry through interaction of the viral envelope proteins with the respective cell surface receptors, reverse transcription of the incoming viral single-stranded RNA to double-stranded DNA and, finally, integration of this DNA into cellular chromosomes (Fig. 4). Thus, as opposed to naked DNA transfection retroviral infection provides the means to control transgene copy numbers and to genetically modify target cells effectively on a permanent basis by inserting genetic cargo into their genome.

In the study of Wei and colleagues,(53) the 5′ end of a Harvey murine sarcoma virus (Ha-MuSV) molecular clone containing the long terminal repeat and the transforming *src* gene was linked to the HSV *tk* gene. Following infection of mouse fibroblast cell clones containing these Ha-MuSVtk recombinant genomes with a replication-competent Moloney murine leukaemia virus (Mo-MuLV) helper, recombination between Ha-MuSVtk and helper sequences restored 3′ genomic termini. These genomes were rendered infectious through their encapsidation into heterologous Mo-MuLV envelopes (pseudotyping). Transduction by recombinant infectious particles was scored by the conversion of TK-negative indicator cells to the TK-positive phenotype. Tabin and coworkers used similar strategies but inserted the *tk* gene between the 5′ and 3′ long terminal repeats of a defective Mo-MuLV provirus clone precluding the need for intermolecular recombination.(54) Shimotohno and Temin employed the conversion of TK-negative to TK-positive rat and chicken indicator cells to demonstrate assembly and transmission of a TK-encoding vector based on the avian spleen necrosis virus.(52) Soon thereafter, the use of replication-competent helper viruses could be eliminated from production protocols due to the development of packaging cell lines. The basic principle of this approach consists of generating stable cell lines containing proviral genomes with the *cis*-acting encapsidation sequences

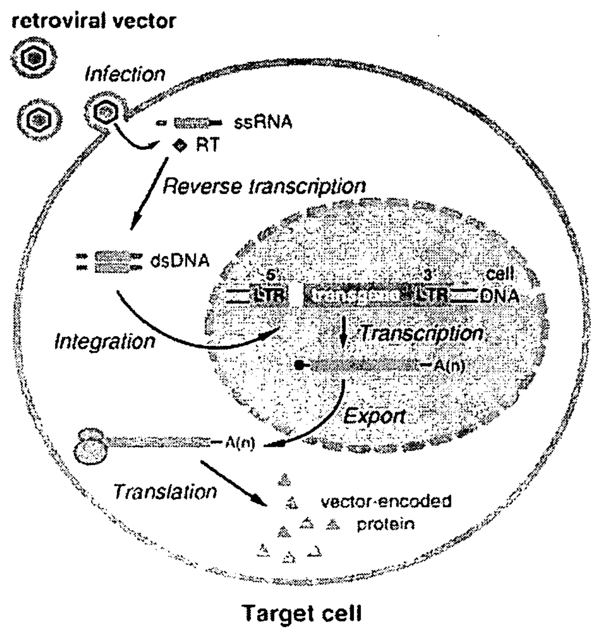

Figure 4. Overview of the retroviral vector transduction process. The foreign genetic information contained in the single-stranded (ss) RNA genomes of a recombinant retrovirus enters into the cytoplasm of target cells through infection, after fusion of the vector envelope with the target cell membrane. The particle-associated reverse transcriptase (RT) converts the ssRNA vector genomes into double-stranded (ds) DNA templates via intermediate RNA–DNA hybrid molecules (not drawn). Upon contact with the cellular genome virus-encoded enzymatic activities catalyse the integration of the recombinant DNA molecules into the chromosomal DNA establishing the stable transduction of the target cell. Vectors based on oncoretroviruses, such as Mo-MuLV, require nuclear membrane breakdown to get access to the cellular DNA, whereas, vectors based on lentiviruses, such as HIV, actively import their reverse transcribed genomes through the pores of intact nuclear membranes. Thus, lentiviral vectors have the distinct advantage of being able to infect non-dividing cells. Whenever integration takes place in a region of the genome favourable for gene expression and gene silencing does not occur, transcription, messenger RNA export and translation of the foreign genetic information into the vector-encoded protein will ensue.

removed. Expression of *gag, env* and *pol* from the resulting packaging-defective templates complements in *trans* the packaging-competent vector genomes.(55)

Initial viral vector-mediated phenotype rescue experiments deployed retroviral vector technology to deliver the human *hprt* cDNA to hypoxanthine-guanine phosphoribosyltransferase (HPRT)-defective cell lines.(56) The first work describing stable transduction of murine haematopoietic progenitors(57) and murine pluripotent haematopoietic cells,(58) used retroviral vectors containing the dominant selectable marker *neo* that confers resistance to the neomycin analogue G418. Joyner and colleagues used colony-forming unit (CFU) assays on G418-containing methylcellulose media to demonstrate that, following in vitro infection of murine BM cells with a retroviral vector, up to 0.3% of granulocyte-macrophage CFU and a few erythroid CFU progenitors, were transduced.(57) The other study by Williams and coworkers used a helper virus-free packaging cell line to produce retroviral vector stocks with high concentrations of infectious particles. Following infection and transplantation of murine BM cells into irradiation-conditioned hosts, the authors looked for gene transfer into a subpopulation of early progenitor cells known to give rise to macroscopic colonies in the spleens of mice (CFU-S) 12 to 14 days post-transplantation. Southern blot hybridizations revealed that nearly 20% of these cells were stably transduced.(58) Shortly thereafter, a series of studies provided conclusive evidence for the stable transduction of bona fide murine HSC as functionally defined by their ability to self-renew and differentiate along the myeloid and lymphoid pathways.(59–62) The non-site-specific integration of retroviral vector sequences into cellular DNA led to specific vector–chromosomal DNA junctions providing researchers with an individual tag to each stably transduced stem cell and its myeloid and lymphoid progenies. These experiments provided conclusive proof of the pluripotentiality of these cells, as previously suggested by studies using radiation-induced chromosomal rearrangements as lineage markers.(63) Improved protocols, namely, (1) depletion of progenitor cells with 5-fluorouracil and consequent enrichment for the non-cycling HSC population,(64) (2) co-cultivation of target and vector producer cells and (3) cytokine treatments to enhance target cell viability with marginal loss of repopulation ability,(65) significantly improved the frequency of animals successfully engrafted with retroviral vector-modified cells.

Following the aforementioned gene marking experiments, retroviral vectors were tested for the delivery and expression of therapeutically relevant human genes, such as those encoding β-globin(66) (haemaglobinopathies), adenosine deaminase (ADA)(67) (ADA-severe combined immunodeficiency [SCID]), and glucocerebrosidase(68) (Gaucher disease). Retroviral vector technology permitted the introduction of a β-glucuronidase-encoding gene into HSC of a mouse model for human mucopolysaccharidosis type VII (Sly syndrome). In this study, the reversal of an inherited disease phenotype was demonstrated for the first time.(69)

In the early 1990s, however, non-human primate and canine preclinical models taught us that the HSC of these larger animals were far more difficult to transduce than their murine counterparts. Despite the use of similar ex vivo retroviral vector-mediated gene transfer protocols, transduction levels as measured by the frequency of transgene-marked rhesus monkey BM and peripheral blood cells approached in the best instances 1%.(70,71) This frequency was more than 10-fold lower than the values achieved in mice. The application of alternative transduction protocols, such as the extended culturing of CD34$^+$ cells (fraction enriched for HSC activity) on a supportive BM stromal layer in contact with vector particles, led to a minor increase in the percentage of transgene-positive cells.(72) With one exception,(73) experiments in dogs also only reached frequencies below 1%. Importantly, in some of these gene marking studies, recipient animals were followed for several years and shown to maintain genetically modified cells from different haematopoietic lineages. Thus, these experiments identified bottlenecks and demonstrated the ability to stably transduce HSC from large mammals.

The elusive character of HSC and their relative inaccessibility to gene transfer led in some instances to the use of more-mature progeny as an alternative target cell source for therapeutic gene transfer. Indeed, the first two human gene transfer attempts using viral vectors targeted lymphocytes. One of them was a gene marking study in which a *neo* gene was transduced to track tumor-infiltrating lymphocytes after their readministration to melanoma patients.(74) The other study was a clinical trial performed to deliver the human ADA-encoding gene to ex-vivo-expanded lymphocytes of ADA-SCID children.(75) This trial commenced in 1990 on the basis of observations showing beneficial effects of allogeneic T-cell transplantation and on the perceived idea that low levels of enzyme in a minor proportion of cells would confer a therapeutic effect. The results published 4 years later documented no deleterious effects and long-term presence of gene-modified cells. Interpretation of therapeutic efficacy, however, was confounded by the continuation of the alternative enzyme replacement therapy.

As predicted from the experiments in large animals, gene marking by infection of human BM cells with retroviral vectors yielded less than 1% gene-modified progeny.(76) The realization that vectors based on simple retroviruses (e.g. Mo-MuLV) are dependent on mitosis to gain access to the target cell DNA,(77) directly hinted that the quiescent status of most of the human and non-human primate HSC imposes a block to transduction. The low levels of retrovirus receptors on human HSC were identified as another problem. Consequently, a plethora of strategies were pursued to increase HSC transduction at the levels of gene delivery and expression. Among these were the treatment of cells with cytokine cocktails to augment the fraction of cycling HSC, the pseudotyping of vector particles with envelope proteins from viruses that use cellular receptors more abundantly expressed at the surface of HSC,(78) the physiological upregulation of retroviral receptors(79) and the testing of other emergent vector systems.(80,81) These attempts had varying degrees of success but, in general, did not provide transduction and expression levels compatible with the correction of most disease phenotypes. Of note, however, was the use of less-cell-cycle-dependent vectors based on lentiviruses (e.g. human immunodeficiency virus [HIV]) to deliver a reporter gene to human cells capable of long-term haematopoietic repopulation of recipient NOD/SCID immunodeficient mice. With this surrogate model of human haematopoiesis, it was demonstrated that an HIV vector transduced so-called NOD/SCID long-term repopulating cells significantly better than a Mo-MuLV vector.(81) Issues related to the pathogenic nature of the parental virus add, however, an ever-present note of caution and will make penetration of lentiviral vectors in human clinical trials dependent on extensive preclinical testing. Nonetheless, the efficient transduction of non-dividing cells and higher genome and particle stability of lentiviral vectors over simpler retroviral vectors allows the rescue of disease phenotypes in an increasing number of animal models.(82)

Although initial attempts at human gene therapy were dominated by retrovirus vector-mediated gene delivery, by the mid 1980s, gene carriers based on the DNA viruses, adenovirus(83) and adeno-associated virus (AAV)(84,85) started to be developed. Approximately one decade later, adenovirus and AAV vectors entered human clinical trials aiming at the delivery of CFTR coding-sequences to cystic fibrosis patients.(86,87) Cumulative data indicate that these two systems have a strikingly complementary set of features. Helper-independent adenovirus vectors (produced in complementing cells expressing the deleted viral genes) can be easily produced, have a relatively large coding capacity and are unsurpassed in their transduction efficiency. However, in immunocompetent animals, transgene expression albeit initially very strong, is short-lived (i.e. generally less than two weeks).(88) In contrast, AAV vectors have been historically difficult to produce, have a limited coding capacity and normally lead to a slow rise in transgene expression. Importantly though, AAV vectors can achieve long-term expression in immunocompetent animals (i.e. years).(88) The different expression profiles achieved with adenovirus and AAV vectors seem to relate to their differential infection of antigen-presenting cells and their different viral gene content. Adenoviral vectors are rendered replication-defective through the elimination of only a reduced number of viral genes, whereas AAV vectors, like retroviral vectors, do not encode any potentially immunogenic viral gene product. For this reason, the latter vector system is gaining momentum in the targeting of pathologic conditions that require permanent transgene expression (e.g. hemophilias) whereas helper-independent adenovirus vectors are becoming specialized in the short-term production of therapeutic proteins (e.g. anti-cancer products and immunogens).

In addition to the adaptation of established vectors to particular conditions, extensive reengineering efforts are also underway. Among these are targeting, "gutless", hybrid and oncolytic strategies.(89,90) Genetic, chemical and immunological approaches to target viral and non-viral vectors to specific cell types are being pursued to maximize systemic delivery and achieve higher therapeutic indexes. Moreover, to increase vector safety and minimize elimination of tranduced cells by the immune system, the latest generation of vectors based on adenovirus and HSV are "gutless" (i.e. devoid of viral genes). Assembly of hybrids is a more-recent strategy aiming at combining the desirable elements from disparate systems. Finally, endowing vectors with the ability to replicate selectively in tumor cells rendering them oncolytic is another line dubbed virotherapy by which viral agents are being exploited. The renewed emphasis on vector development was spurred by the realization during the mid 1990s that clinically useful gene transfer (i.e. actual human gene therapy) would be much more difficult to achieve than anticipated. Perhaps the crescendo of successes of molecular biology gave many scientists confidence that translation of human gene therapy from concept to fruition would come soon. In 1995, several peer-reviewed reports of disappointing results of much-publicized human clinical trials contributed to negate this impression.(1) Assessment of the field by a committee chaired by Orkin and Motulsky concluded that inadequate tools were used in several studies and stressed the need for improved vector designs and regulated gene expression. In 1999, a teenager suffering from a metabolic disorder died after intrahepatic administration of a very high dose of an adenovirus vector.(91) This was the first death directly attributable to a gene transfer procedure and it constituted another setback to a field already baffled by insipient delivery of accumulated expectations.

The turning point came the following year when Cavazzana-Calvo and coworkers published results that can be regarded as a milestone since, for the first time, the word "cure" of a genetic disease by gene transfer could legitimately be applied.(92) Specifically, a classic Mo-MuLV vector was used to transfer ex vivo a gene coding for the common cytokine receptor γ chain (γc) to CD34$^+$ cells from children with X-linked SCID (X-SCID). The absence of a functional *γc* gene leads to a developmental block of T and NK cells. X-SCID constitutes an excellent model for gene therapy. Because the *γc* gene is expressed constitutively throughout the entire haematopoietic system, there is no critical need for strict temporal and spatial therapeutic gene regulation. More importantly, *γc*-transduced cells have a significant selective advantage over unmodified cells and, therefore, a few corrected cells can expand and reconstitute immunity. The great merit of this work was to identify and exploit pathophysiological mechanisms of a disease that are compatible with the limitations of existing gene transfer procedures. Additionally, as initially hoped, recent results indicate that ADA-SCID, despite having a disease phenotype more challenging than that of X-SCID, can also be corrected by retroviral gene transfer.(93) This latest ADA-SCID trial was carried out in HSC as opposed to mature T lymphocytes. Also key to its success seems to have been the withdrawal of bovine ADA protein supplementation thought to have blunted the predicted selective expansion of *ADA*-corrected cells in previous attempts. Because of the growth advantage conferred to *ADA*-expressing cells in the absence of enzyme replacement, ex vivo gene transfer could be combined with a mild, non-myeloablative BM conditioning regimen to favor engraftment of the transplanted cells.

Unfortunately, despite these encouraging results, retroviral vector insertion near to and deregulation of the proto-oncogene *LMO-2* contributed to subsequent overgrowth of T-cell clones in two X-SCID patients.(94) These serious adverse events presented as a leukemia-like syndrome were surprising since the risk of insertional oncogenesis was considered to be negligible based on previous trials and on the perceived, though not universally accepted, notion of random retroviral integration.(95) Contemporary to these results, PCR-based cloning and the recently available human genome sequence allowed genome-wide identification of provirus–chromosomal DNA junctions. These studies established unequivocally the non-random character of retroviral integration since a high prevalence of proviruses localized either in the vicinity of promoters (i.e. Mo-MuLV) or within transcription units (i.e. HIV).(95) However, indications exist that interplay between insertion-mediated *LMO-2* deregulation and specific growth-promoting properties of constitutive *γc* expression may have shortcut an otherwise lengthier multi-hit pathway to malignancy.(96)

Conclusions

Gene therapy surged as an intellectual by-product of fundamental discoveries in molecular biology and evolved as a new branch of experimental medicine fuelled by technological advances in genetic manipulation. Realization of limitations of currently available tools has led to more realistic expectations and pragmatic approaches such as selection of disease targets beyond inherited disorders (e.g. cancer) and fine-tuning the available gene transfer protocols to tractable disease phenotypes (e.g. SCID). Nonetheless, the serious adverse events of the X-SCID trial remind us of the complexities of clinical gene therapy even on the face of initial success. This is further stressed by recent studies deploying AAV vectors to induce factor IX and erythropoietin (Epo) synthesis in hemophiliacs and non-human primates, respectively. Preliminary(97) and published(98,99) data from these experiments reveal immune reactions previously not observed in inbred rodent models. Outstanding among these is the induction of neutralizing antibodies against a self-antigen.(98,99) The latter sobering results are puzzling considering another recently published 6-year follow-up study of rhesus macaques to whom Epo-encoding AAV vectors were administered. In these experiments, autoimmunity did not develop and Epo expression was not extinguished.(100)

Overall, one can conclude that further improvements in gene transfer technologies (e.g. control over transgene expression and integration) and deeper insights in host–vector interactions (e.g. knowledge on vector and gene-modified cell biodistribution following different routes of administration and the impact on innate and adaptive immunity) are warranted before clinical gene therapy reaches maturity. In the path to fruition, outbred large animal models will be ever more instrumental in the safety and efficacy assessment of gene transfer methodologies.

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REVIEW

Gene therapy progress and prospects: targeted gene repair

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The capacity to correct a mutant gene within the context of the chromosome holds great promise as a therapy for inherited disorders but fulfilling this promise has proven to be challenging. However, steady progress is being made and the development of gene repair as a viable and robust approach is underway. Here, we present some of the recent advances that are helping to shape our thinking about the feasibility and the limitations of this technique. For the most part, these advances center on understanding the regulation of the reaction and validating its application in animal models.
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Keywords: *gene correction; mismatch repair; D-loop; double-strand breaks (DSBs); replication forks; single-stranded oligonucleotides*

In brief

Progress

- Homologous recombination is a crucial part of the gene repair mechanism
- Single-stranded DNA catalyzes gene repair most effectively
- *In vivo* applications of gene repair are being assessed

Prospects

- Regulatory pathways will guide experimental design and clinical applications
- An understanding of the regulation of gene repair will help to define the feasibility of this approach and permit combinatorial therapy to take shape
- Oligonucleotide delivery must be optimized before gene repair is considered useful for clinical applications
- The cellular response to synthetic oligonucleotides will be a focal point of intense study
- More animal models must be tested to validate the overall approach

Introduction

Few scientific endeavors have received as much scrutiny during its early stages of development as targeted gene repair. Some of the criticism over the past 2 years has been constructive, and has helped to formulate our thinking about the reality and issues that surround this technique. Over the past several years, emphasis has been placed on elucidating and understanding the mechanism of action in an effort to stabilize the technology and eventually consider it for clinical use. It is our hope that gene repair will not suffer the same fate as other gene therapy applications that have been advanced before their time often with devastating results.[1] This review focuses on recent developments in the area of gene repair directed by oligonucleotides and offers some perspective on the mechanism and its regulation. We also point out a new, but important, issue that has arisen unexpectedly as the power of the technology is harnessed, and begins to exhibit robust and efficient correction.

Homologous recombination activities play a crucial part in the gene repair pathway

The concept of correcting single-base mutations directly in the chromosome is not new. Pioneering work by Sherman *et al* working in yeast, and Kucherlapati *et al* working in human cells (see Liu *et al*[2] and references therein), provided an early glimpse of the remarkable potential of single-stranded DNA in altering genomic sequences. During this period, the field moved forward at a slow pace, primarily because prokaryotes proved to be refractory to these types of approaches and thus, important model systems from bacterial cells were not readily available. Recently, however, Court *et al*[3] introduced the technique of 'recombineering' in bacterial

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chromosomes and bacterial artificial chromosomes (BACS)[4] in which short single strands of DNA are used in conjunction with a protein from bacteriophage λ to alter single nucleotides in the coding regions of genes. Future work in mammalian cells will undoubtedly use this valuable information and capitalize on these advances. However, as it now stands, the progress in this field over the last 2 years has come largely from explorations into mechanisms, with *some* new animal data that might set the stage for clinical applications.

The gene repair pathway is initiated upon the introduction of naked DNA molecules into cells and their homologous alignment with a complementary sequence in a target gene; in some cases, the DNA pairing events creates a structure known as a D-loop (Figure 1).[5] The strands pair in perfect register except for a single mismatched base pair, located in the center of the complex. D-loops in some form serve as reaction intermediates, with the potential for being resolved in several different ways. The resolution ultimately leads to a change in the targeted nucleotide (see below). It is fairly well established that the initialization of the reaction occurs through DNA pairing, a reaction catalyzed by proteins from the homologous recombination (HR) pathway. Our understanding of this phase comes largely from genetic studies in yeast that have revealed a role for proteins such as Rad51, 52, and 54.[6] Igoucheva *et al*[7] demonstrated a role for human Rad51 in promoting gene repair in mammalian cells providing support for the results obtained in lower eucaryotes. Thorpe *et al*[8] reported similar results in mammalian cells by over-expressing hRAD51 in HT1080 cells. Recently, Olsen *et al*[9] showed that gene repair in CHO cells relies heavily on the HR protein, XRCC2. Thus, it now seems clear that DNA pairing, a part of HR pathway, is important for strand assimilation and the start of the gene repair reaction.

The cell normally uses HR to repair DNA damage resulting from double-strand breaks (DSBs) or stalled replication forks. Thus, one might predict that the activation of HR would also induce a higher level of gene correction and this appears to be the case. Ferrara *et al*[10,11] showed that DSBs introduced through the action of the anticancer drugs, etoposide (VP16) or camptothecin (CPT) stimulated gene repair activity in DLD-1 cells. Suzuki *et al*[12] found that the anticancer drug bleomycin, which causes DSBs also stimulates the gene repair reaction by raising the endogenous levels of HR proteins in the cell. The biotechnology company Sangamo has reported that DSBs induced by chimeric zinc-finger nucleases increase gene correction dramatically.[13,14] However, the introduction of breaks in the chromosomal DNA with the transient production of free ends may prove problematic for cellular metabolism and progression through the cell cycle (see below). Exciting data are appearing from labs exploring the use of rare-cutting endonucleases such as I-SceI and zinc-finger/FokI chimeras that can cleave genomic DNA at fixed (and

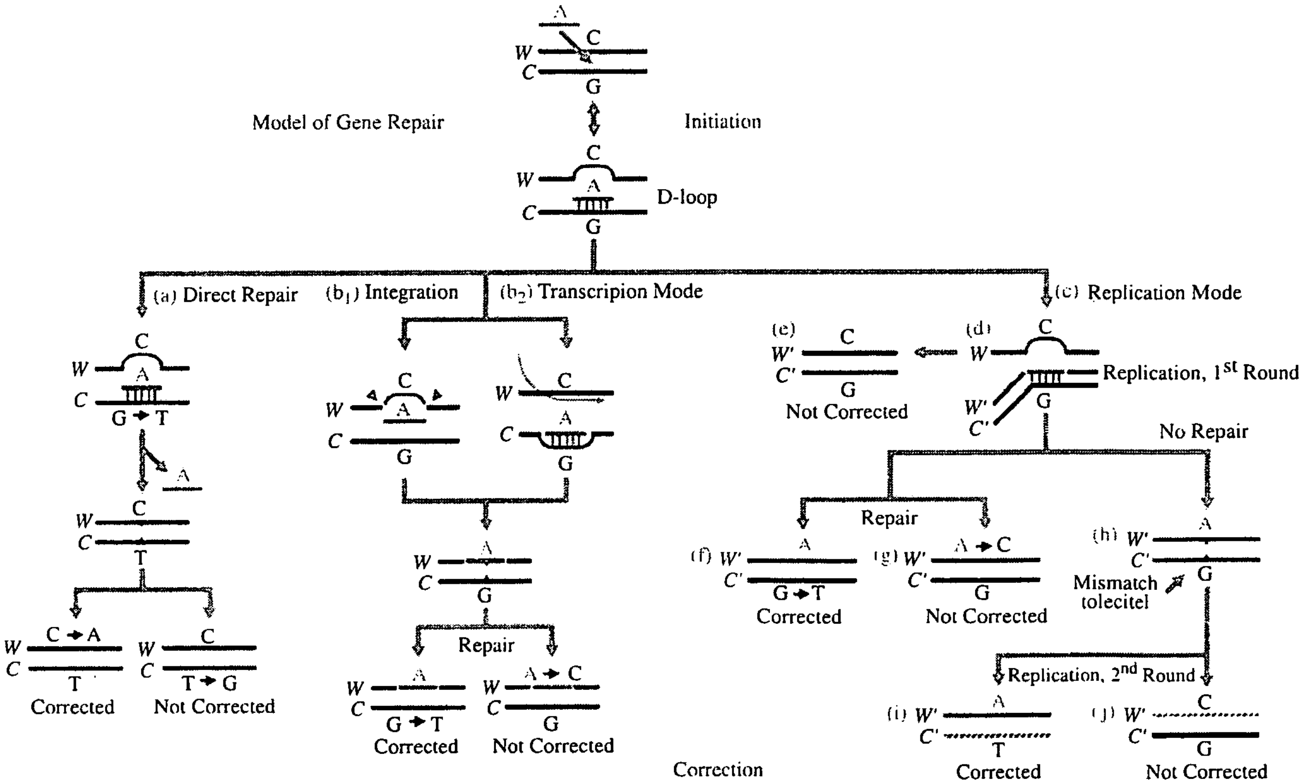


Figure 1 Model of gene repair. This schematic represents three possibilities for the pathway of repair using a single-stranded oligonucleotide (shown as red line). The initiation phase involves homologous alignment of the oligo with its target site, followed by the correction phase, which is discussed in detail in the text. Pathways designated as (a–j) represent possible modes of correction of a mismatch, replication-dependent or replication-independent. The newly replicated strands are shown in blue. Watson strand, W; Crick strand, C.

few) locations.[15–19] It is possible that by minimizing the number of DSBs, the gene repair frequency will be elevated without the concurrent pleiotropic effects.

The events of the second phase where the 'correction' of the targeted mutant gene actually occurs are poorly understood. In this case, unfortunately, genetic data from yeast have not provided a clear picture of this phase of the reaction with some strains deficient in mismatch repair activity (MMR) exhibiting a low number of events, while other strains exhibit high correction frequencies. Studies in mammalian cells have also been confusing with cell-free extracts from certain cell lines deficient in MMR functions displaying low correction events,[2,20] while studies in ES cells deficient in MMR function producing opposite results.[21] While these data appear contrary on one level, there is one unifying pathway that can reconcile some disparate results (see below).

Single-stranded DNA catalyzes gene repair most effectively

It is now clear that modified single-stranded DNA molecules generate the most reproducible and robust results, outperforming the original partially double-stranded structure.[22] Transition to single strands was prompted initially by *in vitro* studies on mechanism carried out by Gamper *et al*, and by results in mammalian cell culture (see Liu *et al*[2] and references therein). Important proof-of-concept experiments have been conducted in model systems where the repair of mutations in eGFP,[8,23,24] LacZ,[25,26] HPRT,[27,28] and retinoblastoma genes,[21] albeit at different frequencies, is effectively directed by a single-stranded vector. Partially duplexed oligonucleotides containing regions of single-stranded character have also been shown to be effective, but do not appear to be as active as the completely single-stranded molecule.[9] Short DNA fragments (SDFs) have also been used for catalyzing gene correction in a process labeled short fragment homologous recombination (SFHR).[29] SDFs are generated as PCR products with a typical length between 100 and 600 base pairs. The mechanism of action is unclear but considering the heterogeneity of the vector, it is not unlikely that it follows a similar pathway as oligonucleotides (see below). As we understand more about how the process is regulated, it may be possible to select vectors that produce the stable and robust correction rates in certain types of cells.[2,20]

In vivo applications of gene repair are being assessed

During the last few years, exciting data have been reported when single-stranded DNA oligonucleotides were tested in animal models. Previous work led to the use of single-stranded DNA vectors in an animal model for the severe kidney disease carbonic anhydrase-deficiency (see Lai and Lien[30] for review). Beyond the successful demonstration of the technique and the reversal of the mutation detected at both the genotypic and phenotypic levels, no nephrotoxicity was found in the treated animals, as assessed by blood-urea nitrogen (BUN) levels and renal histology. Nevertheless, perhaps the most exciting results have come recently from studies using the gene repair technique to correct single-base mutations in liver cells. While some skepticism arose from early reports which reported an abnormally high frequency of gene repair activity in primary hepatocytes, the new data provide a scientifically sound foundation for considering liver cells as a primary target for the clinical applications of gene repair (see Andersen *et al*[20]). Lu *et al*[31] demonstrated a specific nucleotide change and a dramatically altered phenotype directed by single-stranded oligonucleotides in the acid α-D-glucosidase gene in 0.5–4% of cells after tail vein injection. Importantly, both *in vitro* and *in vivo* (mouse) models were used to validate nucleotide alteration, which was confirmed at the DNA and enzymatic levels. These authors utilized an asialoglycoprotein receptor ligand to target hepatocytes, overcoming the problem in the liver. In a more recent study, Nakamura *et al*[32] observed conversion of the transthyretin gene in HepG2 cells and in a transgenic mouse, again clearly confirmed at the DNA and RNA levels. These results have generated considerable excitement and enthusiasm because the levels of conversion (9–11%) have immediate clinical benefits for familial amyloidotic polyneuropathy patients. However, not all animal models generate high levels of correction. Nickerson *et al*[33] have established a LacZ mouse model for testing gene repair activity and initial results were quite disappointing as no correction was detected using a variety of vectors. As portrayed by these authors, the delivery of the oligonucleotide in sufficient numbers to a particular tissue may be a continual problem for this approach.

Prospects: regulatory pathways will guide experimental design and clinical applications

The current challenge centers on the fate of the oligonucleotide paired at the target site and its role in the correction event. From our perspective, this is the most interesting and exciting area for future study as the answers may lead to an understanding of not only how it works but also which cellular factors are involved in its regulation. The phase in which a genetic change occurs should be labeled *'correction'* because the *product* of the overall reaction is a corrected gene, not just an altered nucleotide.

Three possible fates for the oligonucleotide aligned at the target helix are presented below, with some evidence for each pathway (Figure 1). Clearly, the level of transcriptional activity[34] and/or the movement of DNA replication forks[35,36] could influence the route taken by the oligonucleotide. One pathway (Figure 1a) involves a direct nucleotide exchange reaction that could be catalyzed by mismatch repair or nucleotide excision repair activities. In the schematic, the A residue of the oligonucleotide directs the conversion of a T in the Crick (C) strand; this pathway does not require extensive DNA replication and probably no transcriptional activity although both could assist in making the target site more available for oligo binding. The C strand then directs correction of the base in the Watson (W) strand from C→A. The reverse reaction is also possible, wherein the nonconverted gene status persists and no change in the sequence is made. Some evidence from studies in yeast supports a role for mismatch repair in the correction process, but, as stated above, not all the data support this model (see references in Liu *et al*[2] and Dekker *et al*[21]).

In the second scenario (Figure 1b), the oligonucleotide integrates into the W strand directly, then mediates a G→T switch on the C strand. Igoucheva *et al*[37] have proposed a model based on transcriptional activity. This involves DNA pairing, D-loop formation, resection of DNA, integration of the incoming oligonucleotide and repair of the mismatched base pair. This model attempts to explain the well-known 'strand bias' effect; oligonucleotides complementary to the antisense (AS) exhibit a higher level of gene correction than oligos targeted to the sense strand oligonucleotide and repair of the mismatched base pair. Such a model predicts that the AS strand would be heavily favored as the oligo target but recent data argue against this as the sense strand has been shown to be favored in some cases,[38] while no bias has been observed in others.[39] In fact, results from a study using the same LacZ system revealed that the strand bias actually switched when the target site was moved within the LacZ gene.[40] Perhaps, gene repair takes this route with genes that are highly transcribed, and is applicable only for genes driven by a CMV promoter in model systems or highly active promoters in natural genes.

The DNA replication process regulates gene repair activity

Recently, several reports have suggested that DNA replication can strongly influence the overall process of gene repair perhaps at multiple levels.[11,36,41,42] In a passive role, one could envision that DNA replication functions simply to destabilize chromatin around the target site making it more accessible to the oligo. However, replication could play a more direct and dynamic role in the mechanism of correction. In light of the data presented below, we propose a novel pathway in the correction phase called the *replication mode* of gene repair (Figure 1c, right side of the diagram). In this scenario, initiation of the reaction would occur in a similar fashion to the other models as the oligo aligns to the site in the genome and creates a D-loop. In this case, however, the D-loop could be enveloped or bypassed by the replication fork, or may itself act as a signal for the binding of proteins that initiate origin firing (Figure 1d). In either case, when the replication fork meets the D-loop, the oligo would be assimilated into the daughter strand between the Okasaki fragments of the lagging strand or at the 3′ end of the leading strand. In the diagram, the W strand (leading or lagging) that is not bound by the oligonucleotides is simply displaced and replicated fully, generating an unconverted copy of the gene (Figure 1e). However, the other strand contains the annealed oligo with the mismatched base pair complex.

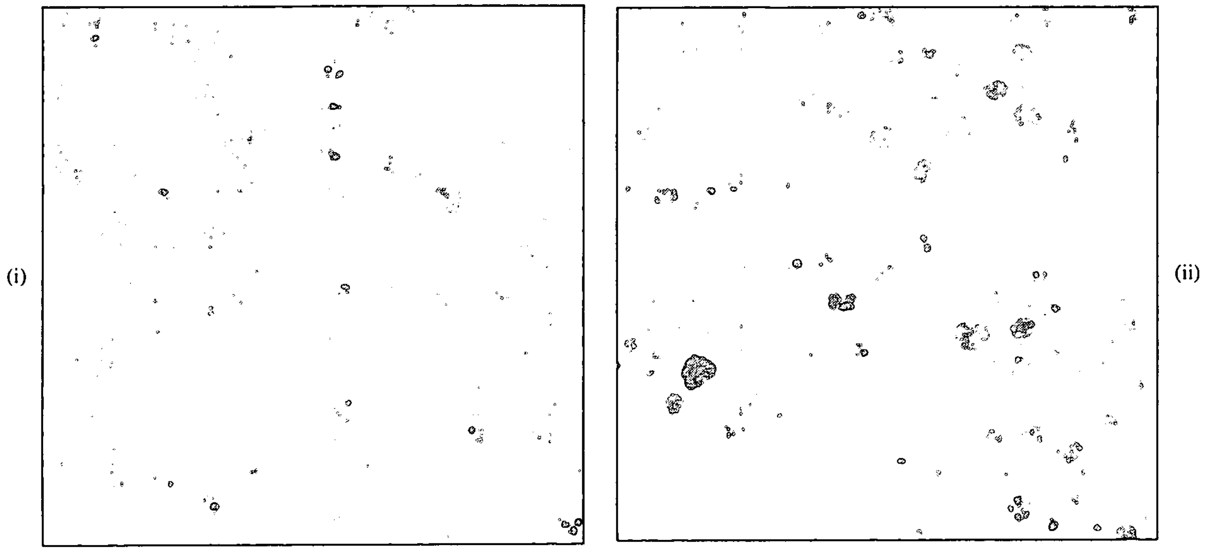
In this example, the A/G mismatch could be repaired in two ways. Firstly, the A directs the conversion of the G residue to T in the C parental strand (Figure 1f). The likelihood of this directional repair is probably low since current views of mismatch repair activity during DNA replication support the notion that the parental strand serves as a template to direct correction on the daughter strand resulting in no correction.[43] In this case, the results would be an uncorrected gene. Secondly, perhaps the most intriguing aspect of this pathway is the structure presented in Figure 1h. If we assume that the mismatch is tolerated through a single round of replication or MMR pathway is inactive, then a second round of replication could generate a corrected gene (Figure 1i) and a parental noncorrected gene (Figure 1j) *without* DNA repair activity. In the final analyses, two rounds of DNA replication yield a converted gene in one of four cells. The important caveat of this scenario, however, is that the mismatched base pair created between the oligonucleotide and the target site is tolerated and is not resolved prior to the strands being replicated.

If this caveat is accepted, then one might predict that cells deficient in MMR would be able to tolerate the mismatch (it would be left unrecognized) and contain an inherently high level of gene repair activity. There is some evidence to support this notion. First, our own studies in yeast demonstrated that strains deficient in some MMR genes such as *PMS1* supported enhanced levels of correction. Second, a mammalian cell system that has been used to characterize gene repair reactions is deficient in hMSH6 protein activity, yet this cell line produces consistently elevated levels of correction.[10] Third, Dekker *et al*[21] found that gene repair in MSH2−/− mouse ES cells is higher than in wild-type cells, and activity in MEF cells deficient in *MSH2* is enhanced as compared to activity in wild-type MEF cells (Drury and Kmiec, unpublished observations). These data lend some support to a long held notion that MMR could act as a suppressor of gene repair by blocking homologous recombination in the form of DNA pairing. MMR proteins inhibit DNA exchange processes between divergent sequences perhaps by binding directly to recombination intermediates. Studies in yeast have clearly demonstrated that the MMR pathway, and *MSH2* in particular, acts specifically to prevent assimilation of foreign DNA into the chromosome, a fundamental requirement of the initial phase of the gene repair reaction. Finally, in many of the gene repair assays, the C/C mismatch is 'repaired' quite efficiently[22,25,44] in contrast to the expected results based on mispair correction hierarchy.[45] This could infer that the replication mode is operational since the C/C mismatch is the least recognized mismatch and in this case, could be tolerated through several rounds of replication. Thus, cells deficient in MMR may enable the gene repair reaction to take place with higher efficiency.

The hybridization of the oligonucleotides to the target site requires a DNA pairing or DNA annealing function most likely provided by the homologous recombination pathway and HR has already been shown to increase the correction frequency. Court *et al*[3] proposed that short oligonucleotides assimilate into the lagging strand of replication forks and direct targeted mutagenesis in bacterial chromosomes and BACS. In view of the recent data, some of which is described above, it may now be appropriate to consider this route as a major pathway of gene repair in eucaryotes. Strand assimilation driven by members of the homologous recombination pathway is activated in response to DNA damage and coincidently would promote the initial phase of gene repair at a higher frequency. DNA lesions induced by drugs such as VP16 or CPT activate HR and retard, stall or inhibit replication.[10–12,41,42,46] All these events elevate gene repair activity but are also subject to downregulation or even suppression by p53 or *MSH2*. While the evidence is sketchy, it is plausible that DNA damage other than simple mispairs could activate the suppressive MMR activity.[43] p53 and *MSH2* have also found to be targeted to sites of replication-associated DNA recombination substrates.[47] It is likely that sites of active gene repair consist of stalled replication forks being acted upon by HR proteins and as such, the colocalization of suppressor functions by MMR could block HR and simultaneously inhibit gene repair events. This model would also predict that cells in S phase are more amenable to gene repair activity and recent data strongly suggest that this is the case.[24,36,42,48]

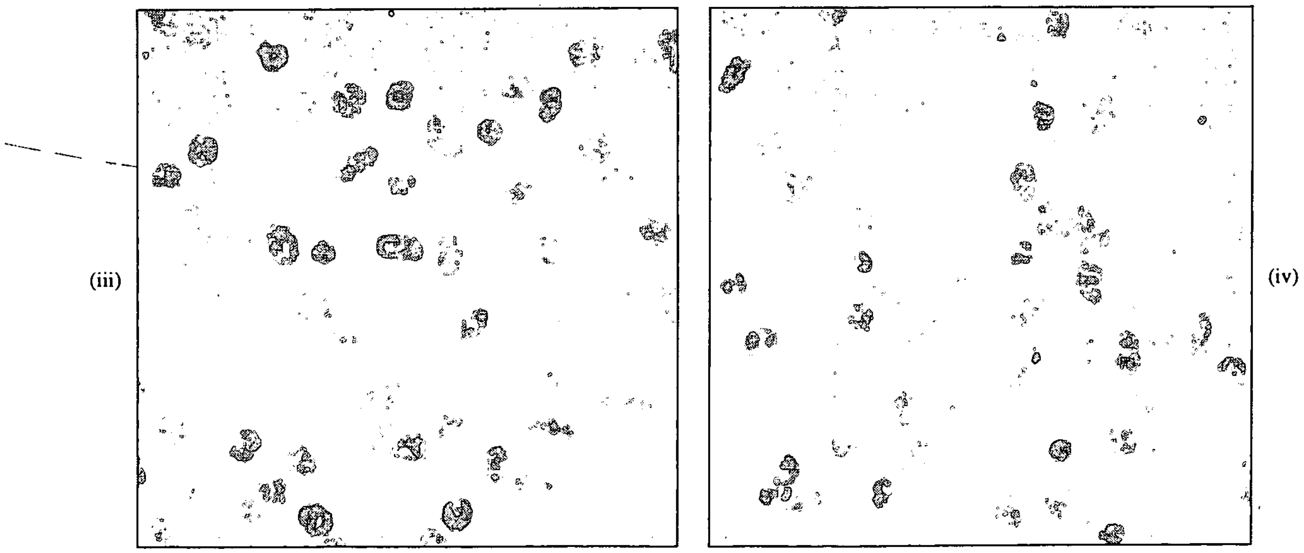
Correction-yes, but at what price?

As more and more systems are developed, the central questions about this technology are changing from does it work and how does it work, to what happens if it works? Our attention has been drawn to the last question by the insightful efforts of Olsen *et al*[9] who made, in our opinion, a critical observation about the inheritance of a corrected mutation. Alexeev and co-workers claimed to be the first to demonstrate permanent genotypic and phenotypic changes in mammalian cells.[37] This report infers that the corrected cells propagate and expand robustly, but, Olsen *et al*[9] found that the percentage of corrected cells *decreases* with time and … 'observed that apoptosis is coupled to the targeted sequence alteration rather than to general toxicity'. Such a phenomenon had been noticed previously but the data were not completely revealing and no clear explanation was offered to explain this observation.[49] However, if one considers the experimental protocols used to introduce the



No oligo

Supercoiled plasmid



Non-specific oligo-Kan/LD50C

Specific oligo for eGFP mutation-EGFP3S/47NT

Figure 2 ssDNA activates ataxia-telangiectasia-mutated (ATM) protein. ATM is activated upon DNA damage to the cells. Confocal images of DLD-1 cells stained with anti-ATM p-S1981 antibody (represented by red fluorescence), 24 h post introduction of the vector via electroporation. (i) electroporated cells without vector (control); (ii) electroporation of a supercoiled plasmid; (iii) and (iv) single-stranded nonspecific (NS) (KanLD50C) and specific (EGFP3S/47NT) oligonucleotides to an integrated eGFP gene, respectively.

oligonucleotides, this type of cellular response should not be surprising but rather predicted. In the gene repair reaction, single-stranded DNA molecules are introduced at a high dosage and their presence obviously creates a molecular environment in which free ends abound. It is widely recognized that free ends induce a DNA damage response, which leads to cell cycle arrest, replication fork stalling and the activation of repair pathways, including HR. The combination of these activities determine the fate of the cell; whether the damage will be repaired or the cells will be programmed for death.[50] In effect, the cellular response to single-stranded vectors that direct gene repair is a double-edged sword. On the one hand, HR proteins are induced and help to catalyze the strand assimilation (perhaps into stalled forks), while on the other hand, their presence activates pathways that monitor genome integrity and could destroy the corrected cell. If true, one might predict that the correcting oligonucleotides would induce either ataxia-telangiectasia-mutated (ATM) or ataxia-telangiectasia-mutated and Rad3-related (ATR) proteins which respond to cellular stress such as a break in the DNA itself or the stalling of a replication fork.[51] Recently, Ferrara *et al* (manuscript submitted) evaluated the induction of ATM in response to the introduction of oligonucleotides into DLD-1 cells. Cells were stained with an antibody directed against phosphorylated (activated) ATM and the data show clearly that the introduction of single-stranded oligonucleotides into a mammalian cell line induces the activation of ATM in contrast to supercoiled plasmid DNA or the electroporation protocol itself (Figure 2). Krauss and co-workers suggest that these corrected cells arrest at the G2/M border,[52] which could result from elevated levels of ATM. Earlier studies examined the effect of single-stranded DNA molecules on mammalian cells, and found that the cell responds to the presence of free ends by arresting cell growth, consistent with this interpretation of the gene repair reaction.[53] Thus, the cell may have to pay a price for achieving high levels of correction and this could explain why the technique has not been as reproducible as predicted from the early reports of success. It may also explain the low or variable levels of correction often obtained in different systems in which corrected cells may have survived to varying degrees prior to analyses.

Will this limit gene repair applications or can we bypass the checkpoint response?

As described above, new studies in animal models report sustained enzyme production as a result of genotypic changes and these alterations appear to be inheritable.[31,32,54,55] One might wonder therefore if the activation of the cellular stress response is only valid for *in vitro* model systems. Reversing the stall by the use of reagents (caffeine) that allow the cells to pass through a G2/M block is one remedy. However, this may be unwise since this natural checkpoint prevents the transfer of unwanted mutations into daughter cells. Perhaps, the most logical strategy is to develop delivery systems that can transfer low amounts of oligonucleotide into the nucleus with high efficiency. It is likely that the movement of the oligo into the nucleus occurs inefficiently using current delivery methods as the oligo may be adsorbed to proteins or routed into terminal complexes. The challenge of oligonucleotide delivery has been the subject of several excellent reviews and the reader is directed to these important papers.[8,32] The authors suggest physical delivery methods such as pluronic gels or nano-particles or the use of signal peptides conjugated to the oligonucleotide that will catalyze transfer to the nucleus. Interestingly, Davis and co-workers introduced several types of synthetic molecules, including the chimeric RNA/DNA oligonucleotide into CD34-positive cells using microinjection and succeeded in demonstrating that the targeted gene was altered precisely.[49] More importantly, the inheritance of the alteration persists as the cells were expanded in culture; a requirement of this approach is to be brought forward into clinic.

Conclusions and the future

Recently, Igoucheva *et al*[56] argued forcefully and we believe, correctly, for the development of better delivery systems and subsequent cellular expansion to achieve long lasting gene repair. This paper takes into account many of the challenges that workers face in this field and demonstrates the type of evaluation that must be undertaken before any disease target can be chosen with confidence. More emphasis needs to be placed on animal models that recapitulate human disease and realistic end points need to be put in place. Thus, gene repair has begun to face the same challenges that serve as barriers for many gene therapy applications, but several unique problems are now being recognized. Moreover, considering the history of this evolving field, one can only hope that these new challenges are met with more open and less frictional mindsets from both inside and outside the field, mindsets that have certainly stalled the development of this potentially important technique.

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to identify the viral sequences required for replication, assembly of viral particles, packaging of the viral genome, and delivery of the transgene into the target cells. Next, dispensable genes are deleted from the viral genome to reduce replication and pathogenicity, as well as expression of immunogenic viral antigens. The gene of interest together with transcriptional regulatory elements (referred to as the transgene) are inserted into the vector construct, and a recombinant virus is generated by supplying the missing gene products required for replication and virion production. The more genes that are removed from the virus, the more replication defective the vector will be, and there is less chance of recombination to generate the infectious parental virus. The nature of the virus biology will usually determine the means of production. For example, retroviruses are produced in packaging cell lines, and vector particles accumulate in the culture medium. In contrast, adenovirus and adeno-associated virus (AAV) vectors are generally produced from transfections, and cells must be lysed to liberate the viral particles. In this review, we describe the salient features and applications of some of the most commonly used viral vectors. There are a number of other emerging vector systems that are still in their infancy and have been extensively discussed in other excellent reviews (1, 3).

RNA VIRUS VECTORS

The most commonly used RNA virus vectors are derived from retroviruses, and these were among the first viral delivery systems to be developed for gene therapy applications. Retroviruses are a large family of enveloped RNA viruses found in all vertebrates, and they can be classified into oncoretroviruses, lentiviruses, and spumaviruses.

Retroviruses

The enveloped virus particle contains two copies of the viral RNA genome, surrounded by a cone-shaped core (for an in-depth review of retrovirus biology, see Reference 4). The viral RNA contains three essential genes, *gag*, *pol*, and *env*, and is flanked by long terminal repeats (LTR). The *gag* gene encodes for the core proteins capsid, matrix, and nucleocapsid, which are generated by proteolytic cleavage of the *gag* precursor protein. The *pol* gene encodes for the viral enzymes protease, reverse transcriptase, and integrase, which are usually derived from the *gag pol* precursor. The *env* gene encodes for the envelope glycoproteins, which mediate virus entry. Oncoretroviruses are simple viruses encoding only the structural genes *gag*, *pol*, and *env*, whereas lentiviruses and spumaviruses have a more complex organization and encode for additional viral proteins (see Figure 2).

After binding to its receptor, the viral capsid containing the RNA genome enters the cell through membrane fusion. The viral RNA genome is subsequently converted into a double-stranded proviral DNA by the viral enzyme reverse

VIRAL VECTORS

The basic concept of viral vectors is to harness the innate ability of viruses to deliver genetic material into the infected cell. In general, the major preoccupation of viruses is to replicate and produce copious amounts of progeny. Most viruses gain little by killing the host, but unfortunately many viral infections lead to deleterious effects on the host, accompanied by destruction of infected host cells. Damaging effects can be caused by induction of genes whose products are hazardous to the host or by acquiring host genomic material that can lead to pathogenesis. The basic principle of turning these pathogens into delivery systems relies on the ability to separate the components needed for replication from those capable of causing disease (see Figure 1). The first step of viral vector design is, therefore,

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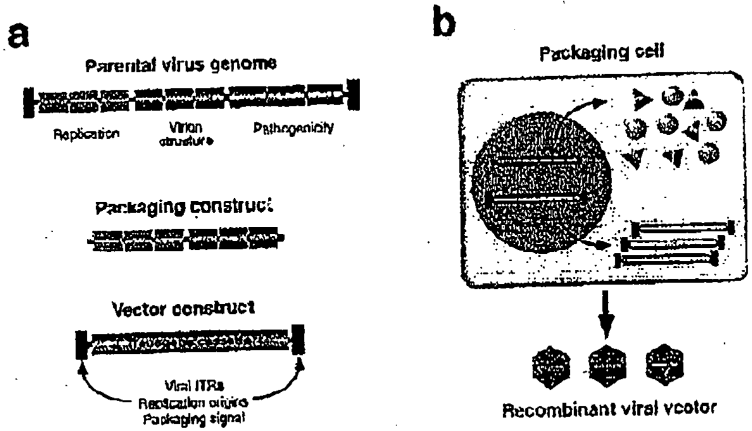

Figure 1 Principle of generating a viral vector. (*a*) Converting a virus into a recombinant vector. Schematic of a generic viral genome is shown with genes that are involved in replication, production of the virion, and pathogenicity of the virus. The genome is flanked by *cis*-acting sequences that provide the viral origin of replication and the signal for encapsidation. The packaging construct contains only genes that encode functions required for replication and structural proteins. The vector construct contains the essential *cis*-acting sequences and the transgene cassette that contains the required transcriptional regulatory elements. (*b*) The packaging and vector constructs are introduced into the packaging cell by transfection, by infection with helper virus, or by generating stable cell lines. Proteins required for replication and assembly of the virion are expressed from the packaging construct, and the replicated vector genomes are encapsidated into virus particles to generate the recombinant viral vector.

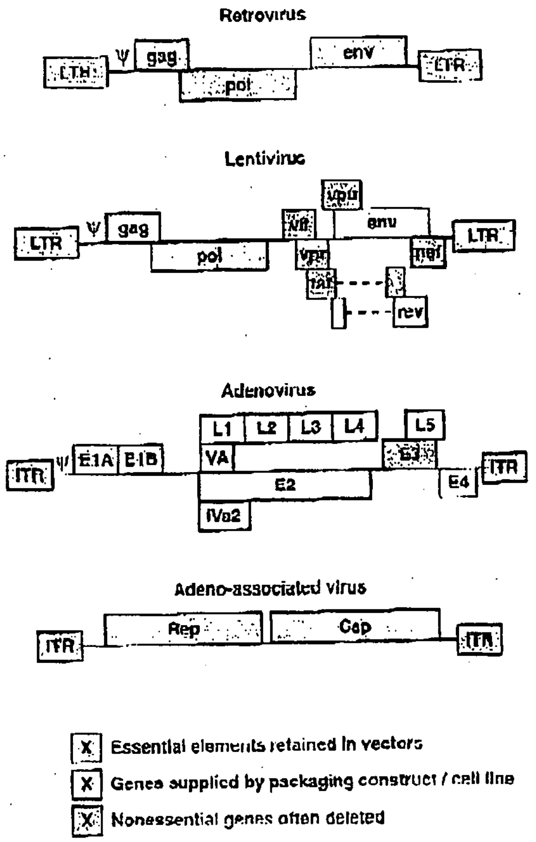

Figure 2 Schematics of the commonly used viruses that are converted to recombinant viral vectors. The colored boxes indicate genes or *cis*-acting elements that are either essential [and therefore retained in vectors (*red*) or supplied by packaging constructs or cell lines (*green*)] or that are nonessential and often deleted (*blue*). Only the major genetic elements are shown, and viruses are not drawn to scale. In second- and third-generation derivatives of each vector system, more viral genes are deleted (see text for details).

transcriptase. The proviral DNA is associated with viral proteins, including nucleocapsid, reverse transcriptase, and integrase, in a preintegration complex and translocates to the nucleus, where the integrase mediates integration of the provirus into the host cell genome. Host cell transcription factors initiate transcription from the LTR, and new viral particles are formed at the plasma membrane. *Gag-pol* and *gag* precursors assemble together with two copies of viral RNA, and *env* glycoproteins are incorporated into the viral membrane during the budding process. In the newly formed virion, *gag* and *gag-pol* precursors are subjected to processing by the viral protease, which results in maturation of the virion.

ONCORETROVIRAL VECTORS These vectors have been derived from a number of different oncoretroviruses, including murine leukemia virus (MLV), spleen necrosis virus, Rous sarcoma virus, and avian leukosis virus. Replication-defective MLV vectors are generated by replacing all viral protein encoding sequences with the exogenous promoter-driven transgene of interest. In addition to the packaging signal, the vectors retain viral LTRs and adjacent sequences, which are essential for reverse transcription and integration. Vector RNA production is either driven by the U3 region of the LTR or by a CMV/LTR hybrid with higher transcriptional activity. In these vectors, the 3′ U3 region of the LTR is intact and is copied over to the 5′ LTR during reverse transcription, allowing efficient integration and LTR-driven transgene expression in the transduced cell.

Packaging of retroviral vectors is achieved by providing the structural proteins in *trans* in the packaging cells. The first packaging cell lines expressed *gag*, *pol*, and *env* from a complete proviral DNA, lacking only the packaging signal (5). However, sequence homology between the vector and packaging constructs facilitated recombination, allowing generation of a replication competent virus. To prevent recombination, packaging cells have been developed that express *gag/pol* and *env* from separate constructs. Furthermore, expression from the packaging constructs is no longer driven by the viral LTR but by constitutive promoters that allow a high level of virus production (5, 6). To avoid the laborious and time-consuming practice of generating cell lines, high-titer vectors can be produced by transient transfection (7). Viruses are recovered from the supernatants of actively growing producer cells.

Major concerns in the use of retroviral vectors are the possibility of vector mobilization and recombination with defective (endogenous) retroviruses in the target cell. This prompted the development of self-inactivating vectors (8). In these vectors LTR-driven transcription is prevented in transduced cells by deletion in the U3, and transgene expression is driven instead by an internal promoter, allowing the use of regulated and tissue specific promoters.

LENTIVIRAL VECTORS In addition to *gag*, *pol*, and *env*, lentiviruses encode three to six additional viral proteins, which contribute to virus replication and persistence of infection (for in-depth reviews of lentiviruses, see References 9 and 10). Two of the accessory proteins, *tat* and *rev*, are present in all lentiviruses and mediate gene therapy is simple: introduce into target cells a piece of genetic material that will result in either a cure for the disease or a slowdown in the progression of the disease. To achieve this goal, gene therapy requires technologies capable of gene transfer into a wide variety of cells, tissues, and organs. One of the biggest stumbling blocks to successful widespread application of such genetic treatments is the development of safe and effective vectors with which to ferry genetic material into a cell.

The process of gene delivery and expression is known as transduction. Successful transduction requires overcoming a number of obstacles that are common to all vector systems (2). The first issue to be addressed is that of production. An ideal vector should be one that can be produced in a highly concentrated form, using a convenient and reproducible production scheme. This has been a challenge for many of the currently used vector systems, but in many cases creative approaches have overcome this barrier. The vector must be capable of targeting the cell type most appropriate for the disease, whether it be dividing or nondividing cells. Understanding of the transduction process through studies of vector uptake, intracellular trafficking, and gene regulation has facilitated the development of efficient vehicles for gene delivery. In many cases it would be desirable to achieve stable, sustained gene expression, which requires either integration of the vector DNA into the host DNA or maintenance as an episome. When using integrating vector systems, it is important to consider the potential hazards of insertional mutagenesis, and thus vectors capable of site specific integration will be attractive. In many cases, expression of the therapeutic gene will require exquisite regulation, and thus the transcriptional unit must be capable of responding to manipulations of its regulatory elements. Finally, no pathogenic or adverse effects should be elicited by vector transduction, including undesirable immune responses.

Vectors that have been developed to overcome these obstacles fall into two broad categories: nonviral and viral vectors (1). The nonviral vectors consist of naked DNA delivered by injection, liposomes (cationic lipids mixed with nucleic acids), nanoparticles, and other means. Although nonviral vectors can be produced in relatively large amounts and are likely to present minimal toxic or immunological problems, presently they suffer from inefficient gene transfer. In addition, expression of the foreign gene tends to be transient, precluding their application to many diseased states in which sustained and high-level expression of the transgene is required. It is likely that future gene therapy protocols will use novel innovations to improve on the efficiency of nonviral vector systems, often building upon observations from viral vector transduction. Viral vectors are derived from viruses with either RNA or DNA genomes and are represented as both integrating and nonintegrating vectors. The former holds the promise of lifelong expression of the deficient gene product. Efficient gene transduction can also be achieved from vectors that are maintained as episomes, especially in nondividing cells.

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GENE THERAPY: Twenty-First Century Medicine

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Key Words viral vectors, retrovirus/lentivirus, adenovirus, AAV, clinical trials

■ **Abstract** Broadly defined, the concept of gene therapy involves the transfer of genetic material into a cell, tissue, or whole organ, with the goal of curing a disease or at least improving the clinical status of a patient. A key factor in the success of gene therapy is the development of delivery systems that are capable of efficient gene transfer in a variety of tissues, without causing any associated pathogenic effects. Vectors based upon many different viral systems, including retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses, currently offer the best choice for efficient gene delivery. Their performance and pathogenicity has been evaluated in animal models, and encouraging results form the basis for clinical trials to treat genetic disorders and acquired diseases. Despite some initial success in these trials, vector development remains a seminal concern for improved gene therapy technologies.

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INTRODUCTION

Gene therapy is a form of molecular medicine that has the potential to influence significantly human health in this century. It promises to provide new treatments for a large number of inherited and acquired diseases (1). The basic concept of

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transactivation of viral transcription and nuclear export of unspliced viral RNA, respectively. Although vectors based on Simian, Equine and Feline lentiviruses have been developed (11), we focus on human immunodeficiency virus type 1 (HIV-1)-based vectors because they have been most extensively studied.

In addition to *gag*, *pol*, and *env*, HIV-1 encodes six accessory proteins (*tat*, *rev*, *vif*, *vpr*, *nef*, and *vpu*). The retroviral vector design provided an excellent template for development of lentiviral vectors. The HIV-1-based lentiviral vectors are devoid of all viral sequences apart from essential *cis*-acting sequences, including the LTRs and the packaging signal (see Figure 2). The *rev* responsive element (RRE) is also included in the vector RNA. The viral *rev* protein is provided in *trans* to ensure efficient nuclear export of the full-length viral RNA genomes through binding to the RRE. Initially the endogenous LTR was used to drive vector RNA expression via transactivation by the *tat* protein (12), but further generations of the vector utilize a CMV/LTR hybrid promoter, which increases vector production and allows vector production to be independent of *tat* expression (13). The accessory genes, *vif*, *vpr*, *nef*, and *vpu*, are dispensable for lentiviral vector production and transduction, and they are deleted from the packaging construct (13). The central poly purine tract (cPPT), from the *pol* ORF, can be included in *cis* to improve nuclear import of the proviral DNA and hence accelerate transduction (14, 15). Furthermore, the biosafety of vectors is improved by the development of self-inactivating vectors, which are less likely to be mobilized following infection with HIV (16, 17).

The HIV-1 *env* glycoprotein has a highly restricted host range in that it infects cells containing CD4 and coreceptors. To broaden the host range of lentiviral vectors, they can be pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) *env*, which is provided in *trans* and imparts a wide tropism (12). Vectors are harvested from the supernatant, and those pseudotyped with VSV-G can be concentrated to produce high-titer preps. Titers can be determined using assays that measure the amount or activity of proteins incorporated in the vector particles, such as the p24gag ELISA assay. Stable packaging cell lines have now been developed, in which the producer cells express the structural proteins from minimal packaging constructs and expression is driven by an inducible promoter to minimize the toxicity of the VSV-G envelope protein (18, 19). Other viral glycoproteins have also been used to pseudotype lentiviral vectors and provide altered cell tropism (20, 21).

FOAMY VIRAL VECTORS Foamy viral (FV) vectors have recently been developed and are quite similar to retroviral and lentiviral vectors (22). The FV genome contains three additional ORFs (*tas/bel1*, *bel-2*, and *bel-3*), with *tas/bel1* being the coactivator of viral transcription (23). In addition to the packaging signal that consists of the 5′-untranslated region and the 5′ portion of the *gag* ORF present in all retroviral vectors, FV vectors contain the 3′ region of the *pol* ORF, which is critical for efficient packaging of these vectors. Similar to other retroviral vectors, the 5′ U3 region of the LTR in the vectors' plasmid can been replaced by a CMV promoter, which increases vector expression and makes vector production independent of *tas/bel1*.

FV vectors are produced by transient transfection of the vector construct as well as the packaging constructs encoding for the structural proteins *gag*, *pol*, and *env* in 293T cells (22). Because the FV envelope has a broad cellular host range, it is used by the vector, and therefore, the *env* sequences are included in the packaging construct or expressed from a separate construct. The FV *env* contains, in contrast to other retroviruses, an ER sorting signal, which allows FV particles to bud from intracellular membranes, and therefore, the majority of the infectious virions is cell associated (24). Consequently, the infectious particles have to be released from the packaging cells by a freeze-thawing process.

Because retroviral vectors were some of the first to be utilized for gene delivery, they have been extensively used in many applications (2, 25). Their ability to integrate and provide long-term gene expression has made them particularly useful for generating stable cell lines that express a transgene of choice and for marking studies of cell lineage. Their dependence upon cell division has restricted in vivo applications to gene delivery in actively dividing tissues, such as stem cells and cancer cells. In contrast, lentivirus vectors have been used for gene delivery in vivo by direct administration in many organs, including brain, eye, liver, and muscle (26). They have also been used for ex vivo transduction of hematopoietic cells, followed by bone marrow transplantation (27).

The limited cellular tropism of the natural envelope of wild-type viruses is one of the barriers for retroviral transduction. However, retroviruses have the ability to incorporate *env* glycoproteins from related as well as unrelated viruses, thus allowing pseudotyping with alternative glycoproteins. A number of different envelopes have been used to generate pseudotyped retroviral vectors with broad host ranges, including the VSV-G glycoprotein or the amphotropic MLV envelope. Pseudotyping also allows transfer of specific tropisms to the vector. Neurotropism and retrograde axonal transport was accomplished by the vector via pseudotyping with the G protein of Mokola lyssaviruses (28), and the filovirus (Ebola Zaire) envelope supported transduction of airway epithelia (21). Interestingly, the entry pathway of the retroviral vector has minimal effect on the transduction efficiency.

Reverse transcription and nuclear translocation of the preintegration complex are thought to be limiting steps in retroviral transduction, especially in terminally differentiated postmitotic cells. Proviral DNA synthesis of all retroviruses depends strongly on cellular conditions, and low nucleoside pools or absence of cellular cofactors might explain the incomplete reverse transcription in quiescent or stationary cells. In contrast to other retroviral vectors, FV vector particles can contain fully reverse-transcribed viral DNA, owing to activation of the process of reverse transcription before virus assembly. This suggests that FV vector gene transfer might be more efficient in certain postmitotic cells in which reverse transcription is limited.

Because HIV-1 is a human pathogen, there are biosafety concerns about the use of HIV-1-based lentiviral vectors. The current HIV-1 lentiviral vector system is depleted of all accessory proteins, and viral sequences in the vectors have been minimized. Thus, the replication of these vectors is highly disabled, and the possibility of homologous recombination is minimized. In addition, codon optimization of the packaging construct further decreases the risk of homologous recombination. The use of vectors based on other lentiviruses might eliminate some of the concerns (11); however, the risk associated with the introduction of nonhuman lentiviral vectors in human tissues is unknown.

DNA VIRUS VECTORS

Of the vectors derived from viruses with DNA genomes, the most prominent are those based on adenovirus (Ad) and the adeno-associated virus (AAV). Adenoviruses contain a double-stranded DNA (dsDNA) genome of ~36 kb, whereas AAVs consist of a single-stranded DNA molecule that is relatively small (~4.7 kb). The basic principals of vector design (Figure 1) also apply to vectors derived from DNA viruses. However, there are important practical differences in terms of construction, production, and purification of these vectors.

Adenovirus

Ads have been isolated from a large number of species and tissue types (for an in-depth review of Ad biology see Reference 29). The human Ad family consists of more than 50 serotypes that can infect and replicate in a wide range of organs, such as the respiratory tract, the eye, urinary bladder, gastrointestinal tract, and liver. The Ad genome consists of a double-stranded linear DNA molecule (size: ~36 kb) with overlapping transcription units on both strands. Extensive splicing results in the production of over 50 proteins; 11 of which are structural virion proteins. The viral life cycle occurs in an early and a late phase, divided by the onset of viral DNA replication. Adenoviral genes fall into three major groups, depending on the time course of their expression during the viral replicative cycle: early (E1A, E1B, E2, E3, and E4), delayed (IX and IVa2), and the major late transcription unit (see Figure 2). The latter is processed into five mRNAs (L1–L5) that share the same carboxy terminus. These transcription units are transcribed by the cellular RNA polymerase II, whereas the viral-associated (VA) RNA is transcribed by RNA polymerase III. The viral genome contains two identical origins for DNA replication within each terminal repeat. The E2 region encodes proteins required for replication, including the viral polymerase, and proteins from the E1 and E4 regions also contribute to efficient DNA replication. The gene products of the E3 region are involved in immune surveillance and suppression but are nonessential for infection in vitro.

The Ad genome is packaged in a nonenveloped icosahedral protein capsid. The fiber protein projects from the virion, and the carboxy-terminal knob domain forms a high-affinity complex with a host cell surface receptor protein. For the majority of Ad serotypes this receptor is the Coxsackie-adenovirus receptor (CAR) (30). In addition to attachment, efficient virus internalization requires an interaction between the viral penton base and the cellular integrin α_V receptor (31). After entry, the virus rapidly escapes from the endosome, and transport to the nucleus is accompanied by gradual disruption of the virus particle (32). The viral genome is imported through the nuclear pore and associates with the nuclear matrix to facilitate initiation of the primary transcription events (33). The Ad genome is transcribed and replicated at discrete replication centers in the nucleus of the infected cell (34), and the viral DNA does not normally integrate into the host genome.

Adenoviral infection causes an initial nonspecific host response with synthesis of cytokines (tumor necrosis factor as well as interleukin 1 and 6), followed by a specific response of cytotoxic T lymphocytes directed against virus-infected cells that display viral peptide antigens (29). In addition, there is activation of B cells and the necessary CD4-positive T cells, leading to a humoral response. Serologic surveys found antibodies against Ad serotypes 1, 2, and 5 in 40% to 60% of children. The immune response of the host against adenoviral proteins is the major hurdle to the efficient and safe use of adenoviral vectors.

Most adenoviral vectors are derived from Ad serotype 5; however, Ad vectors have also been generated from other serotypes, including human Ad2, Ad7, and Ad4 as well as nonhuman viruses. Replication-defective Ad vectors are designed by replacing crucial adenoviral coding regions (35). In the first generation of Ad vectors, the E1 gene was replaced with the transgene (36). Because E1A is the principal protein that activates the expression of other Ad transcription units' genes (37), and other E1 proteins play crucial roles in viral replication, these vectors are replication defective on most cell lines. E1-deleted vectors can be propagated in cell lines that provide the E1 gene products in *trans*, such as the human 293 cell line (38). Transgenes of up to 4.7–4.9 kb can be incorporated into E1-deleted adenoviral vectors (39). The cloning capacity of Ad vectors can be further increased by deletion of additional dispensable sequences from the Ad genome, such as the nonessential E3 region (40). Combining the E1 and E3 deletions provides a total cloning capacity of 8.3 kb in one mutant virus. Newly developed E1-complementing cell lines with a minimal amount of viral sequences help reduce the chances of homologous recombination between the vector and the host genome, and thus, less replication-competent Ad is generated (41, 42). Vectors can be produced at titers of up to 10^{13} particles/ml and are purified by cesium chloride gradient ultracentrifugation or column chromatography.

Although E1-deleted viruses are defective for replication, in some cell types they can produce virus proteins that serve as foreign antigens to induce a cellular immune response. Many attempts have been made to reduce immunogenicity by engineering the second generation of Ad vectors that are additionally deleted in other viral transcription units, such as E2 and E4. Because the E2 region encodes proteins that are essential for replication of the viral chromosome, it has to be provided in *trans* in the packaging cells. A number of deletions have been introduced into the E4 region, which encodes proteins required for efficient viral DNA replication and late protein synthesis. Partial deletions of the E4 region can produce viable vectors, and E4-complementing cell lines have been developed for more extensive deletions (43–46). Although deletions in the E4 region increase the cloning capacity of the Ad vector, some reports indicate that the E4 region may exert a positive effect on long-term expression (47–49).

Theoretically it should be possible to reduce viral genes to a minimum and create "gutted" vectors that carry no viral sequences, apart from the inverted terminal repeats (ITRs) and the *cis*-acting packaging signal (50). These vectors require helper viruses for propagation, generating a problem in the purification of a helper-free virus. An important step toward a third generation of Ad vectors was the development of high-capacity, helper-dependent vectors based on the Cre/*loxP*-system of site-specific DNA excision (51–53). Using the Cre*loxP*-system, 25 kb of adenoviral genome can be deleted from an Ad vector containing *loxP* sites in 293 cells stably expressing the Cre recombinase (52). Alternatively, the packaging signal can be deleted from the Ad helper virus (51, 54). These vectors have a great potential for efficient gene delivery and long-term gene expression, although production is very laborious and the incoming virus particles can still stimulate a deleterious immune response (50, 55).

Adeno-Associated Virus

AAV is a nonpathogenic human parvovirus (for an in-depth review of parvovirus biology see Reference 56). Productive AAV infection requires helper functions that can be supplied by coinfection with helper viruses, such as Ad and herpesvirus. AAV can also replicate in cells that have been put under stress, such as irradiation or treatment with genotoxic agents. In the absence of a permissive environment that will support AAV replication, the viral DNA can become integrated into the host chromosomal genome to establish a latent infection (reviewed in Reference 57).

Many different serotypes of AAV have been isolated, and the list continues to increase (58–63). They have in common a similar size and genomic configurations of replication and structural genes. AAV serotype 2 (AAV2) is the best characterized and has been the most frequently employed recombinant AAV (rAAV) vector. AAV virions are small nonenveloped particles (20–25 nm) that carry a linear single-stranded DNA (ssDNA) genome, which is approximately 4.7 kb in size. There are 2 viral ORFs, *rep* and *cap*, flanked by T-shaped ITRs. The ITRs are important for replication, packaging, and integration, and these are the only genetic elements from the virus that are retained in rAAV vectors (see Figure 2). There are four regulatory Rep proteins that are required for replication and packaging. The *cap* ORF encodes for three structural proteins (VP1, VP2, and VP3) that form the capsid.

The different serotypes of AAV utilize a variety of approaches for cell entry, and this results in different host ranges. The primary attachment site for AAV2 is the ubiquitous heparan sulfate proteoglycan (64). The fibroblast growth factor receptor 1 and integrin $\alpha_v\beta_5$ have both been implicated as coreceptors that facilitate internalization by endocytosis (65, 66). AAV4 and AAV5 use sialic acid, although different carbohydrate linkages determine specificity (67) and the PDGF receptors are also involved in AAV5 infection (68). After binding to its receptor, the virus enters the cell through receptor-mediated endocytosis and is subsequently transported to the nucleus (69, 70). Viral uncoating in the nucleus releases the single-stranded genome that then needs to be converted to a double-stranded form to enable gene expression.

The self-complementary sequences in the ITRs fold back on themselves to form hairpin structures that contain the replication origins. The large viral Rep proteins bind to a specific sequence within the ITRs and mediate replication (56). In addition, the virus relies upon the host cell replication machinery as well as helper functions supplied by the coinfecting virus. The helper proteins promote initiation of transcription, viral gene expression, and DNA replication. Replication proceeds at discrete sites in the nucleus (71), and strand displacement produces both plus and minus genomes that are packaged with equal efficiency into the icosahedral capsid.

The AAV viral vector system was initially developed by replacing the viral *rep* and *cap* genes with transgene sequences. The vector genome can be rescued by supplying Rep and Cap proteins in *trans* from a packaging plasmid, together with a helper virus infection (72, 73). Production of wild-type virus by homologous recombination has been minimized by reducing overlap between the vector and packaging plasmids. Although high-titer AAV vectors can be produced using this system, wild-type Ad contaminates these vector preparations. Knowledge of the helper requirements from the Ad genome enabled the development of plasmid-based systems in which transfection of mini-Ad plasmids into 293 cells (expressing Ad E1 proteins) supplies all the helper proteins (E2A, E4, and the VA RNA) without production of infectious Ad (74, 75). Alternative approaches include generating packaging cell lines or recombinant viruses (either Ad or HSV-1), containing elements from AAV required for replication and packaging (76–78). Baculoviruses have also been developed for production of vectors on large scales in insect cells (79). Knowledge of the receptors has aided the development of column chromatography approaches to purification for different serotype vectors.

AAV vectors based on the serotype 2 capsid have been the most commonly used for gene therapy studies and have demonstrated transduction in a large number of cell types and experimental model systems (reviewed in References 80, 81). The vector can transduce nondividing cell types and has been used in muscle, retina, brain, liver, and lungs. There is initially a slow rise in gene expression levels over the first few weeks after in vivo administration, and then a stable plateau is reached (82). The exact reason for this delay in gene expression is not exactly clear. It may reflect requirements for cytoplasmic trafficking, vector uncoating, and conversion of the incoming ssDNA genome into a dsDNA form capable of gene expression. This step is mediated by the host cell machinery and probably occurs by second-strand synthesis. AAV transduction can occur independently of the cell cycle; however, transduction efficiency is markedly improved in cells during S phase (83). Furthermore, activation of the cellular DNA repair machinery also supports second-strand synthesis, thus improving AAV transduction (84, 85). Some of the alternative serotype capsids give quicker transduction than AAV2 vectors (86). Transduction can also be obtained with faster kinetics using vectors with genomes half the size of wild type, which are thought to reanneal through self-complementation, independently of DNA synthesis (87).

One of the major limitations for the use of AAV as a gene delivery vehicle is the relatively small packaging capacity. The unique ability of AAV vectors to become joined into concatamers by head-to-tail recombination of the ITRs has been exploited as a means to increase the coding capacity (88). In this approach, either the gene itself or the different elements of the transgene expression cassette are split over two AAV vectors that are administered simultaneously (89, 90). Transgene expression is obtained only after recombination between the two viral genomes, but the efficiency is often reduced as compared to single vector transduction.

The AAV vectors do not contain any viral coding regions, and therefore, there is no toxicity associated with gene expression, However, a single injection of AAV vector elicits a strong humoral immune response against the viral capsid, which will interfere with readministration of the vector (91, 92). Furthermore, natural infections have resulted in a high prevalence of circulating neutralizing antibodies against AAV in the majority of the population, which may inhibit transduction. The use of AAV vectors containing cap proteins from different serotypes may overcome the problems of neutralizing antibodies (91), as may modifications to the virus capsid (93).

Herpesvirus

Human herpesviruses are a class of large DNA viruses with double-stranded genomes capable of accommodating a large amount of foreign DNA (for an in-depth review of herpesvirus biology see Reference 94). Herpes simplex virus type 1 (HSV-1) has been developed as a vector for gene delivery (95). The HSV-1 virion is about 20 nm in diameter and consists of four components: envelope, tegument, capsid, and viral genome. The envelope is derived from the cellular membrane and contains approximately 12 viral glycoproteins essential for viral entry. The tegument is the protein layer between the capsid and the envelope, and this layer contains at least 10 viral proteins, which are involved in the shutoff of host protein synthesis as well as in the activation of immediate early viral gene expression and assembly functions. Among these are proteins called VP16 (essential for trans-activation and virion envelopment), VP22 (membrane translocation domain), and virion host shut-off (vhs) protein. The icosahedral capsid consists of seven viral proteins and contains the linear dsDNA genome, which is 152 kb in size and is divided into unique long (U_L) and unique short (U_S) regions that are flanked by terminal repeats. The virus encodes at least 80 viral proteins with very little splicing of genes. A natural infection can result in either lytic replication in mucosal or epithelial cells or in a latent state in neurons with persistence of the virus genome (94).

Four of the HSV-1 glycoproteins in the virus envelope mediate interaction with the host cell and virus entry. The gB and gC proteins bind to heparan sulfate on the cell surface, and this is followed by gD binding to cellular receptors known as herpesvirus entry mediators HVEM/HveA and HveC/nectin-1. The gB, gD, and gH/gL complexes are all thought to be required for fusion of the virus envelope with the cell membrane to allow release of the nucleocapsid into the cellular cytoplasm. During the lytic replication cycle, the host cell protein synthesis is shutoff immediately after infection by the tegument vhs protein. Subsequently, the viral capsid releases the viral DNA into the nucleus, where it will circularize. Within several hours after infection, protein expression from the circular genome is initiated. Protein expression occurs in a highly regulated fashion and can be divided into three groups of sequentially expressed proteins: α- or immediate early genes, β- or early genes, and γ- or late genes. Once β-gene products are present, DNA replication and γ-gene production are initiated, and progeny virus will be produced. In the nucleus, capsid proteins assemble together with the viral DNA genomes, and the newly formed viral capsids bud through the nuclear membrane. On their way to the Golgi apparatus, the virion obtains the tegument and the viral envelope, and subsequently the virus is released from the cell through secretory vesicles.

Virus replication in the permissive epithelial cells produces virions that can enter the nervous system through axon terminals of local sensory neurons to enable establishment of a latent infection. Upon entry, the virus nucleocapsid is transported to the nucleus in the cell body by retrograde transport along the axon. The viral genes, which are involved in the establishment of a latent infection, are currently unknown, but de novo viral protein synthesis is not required. However, latency is related to the expression of latency-associated transcripts (LAT), which are expressed from a promoter that is highly active in neurons (96). The virus persists in the latent state for the lifetime of the infected individual. Reactivation of the latent virus can be induced by different stimuli, such as stress and UV irradiation, and results in production of progeny virions that are transported along axons to establish an active infection at the primary site of infection.

Replication-deficient HSV-1 vectors have been developed that do not express lytic genes and can result in quiescent and persistent genomes in neurons (97). It is relatively easy to manipulate and engineer the virus genome by recombination to insert transgenes and delete virus genes involved in lytic growth or toxicity. Two different viral vector systems have been developed to generate either recombinant HSV-1 vectors or HSV-derived amplicon vectors. Recombinant HSV-1 vectors contain a number of deletions in the α-genes and the vhs, and they can harbor large transgenes up to 30 kb in size (98). These vectors are nonreplicating and can be propagated to high titers in complementing cell lines that provide the essential α-genes in *trans*. Because many of these virus genes are toxic, they are often expressed in an inducible fashion in complementing cells. Recombinant HSV-1 vectors still retain large proportions of the HSV-1 genome and can express viral genes that induce cytotoxicity and immune responses. Moreover, transgene expression by recombinant HSV-1 vectors is usually transient. It may be possible in the future to harness two viral latency-active promoters to provide stable gene expression from HSV-1 vectors.

The HSV-1 amplicon vector system is based on the ability of HSV-1 to package defective genomes containing the *cis*-acting sequences *ori* (origin of viral DNA replication) and *pac* (packaging and cleavage signal). Besides these *cis*-acting elements, no other viral genes are retained in HSV-1 amplicon vectors (99). However, packaging of the amplicon vectors requires a replicating helper virus, which can result in high-level contamination with a replication-competent virus. This problem has been overcome by the development of a helper-free packaging system in which viral genes are provided in *trans* from cosmids or from a bacterial artificial chromosome lacking the *pac* signal.

Transduction with HSV vectors has been demonstrated in a large number of cell types, and these vectors have been applied to multiple gene therapy strategies, including neurological diseases, spinal nerve injury, glioblastoma, and even pain therapy (95). Sensory neurons can be infected by direct interdermal injection of the vector, and the DNA can persist in the nerve cell body. Maintaining high gene expression levels over long periods of time is a problem in certain cell types, such as the brain. The major limitations for recombinant HSV-1 vectors are their cytopathic effect and the induction of an immune response by viral gene expression. The development of amplicon vectors and a helper virus-free packaging system has overcome this problem (99). However, additional deletion of nonessential genes from the *bac* packaging system may also be necessary to prevent cytotoxicity and recombination within this vector system. The large packaging capacity of HSV-1 amplicons (up to a theoretical 152 kb) may be very useful for delivering complex genes and regulatory sequences or multiple copies of the transgene.

VECTOR TROPISM AND THE SPECIFICITY OF TRANSDUCTION

Although recombinant vectors have demonstrated wide host ranges, this may be disadvantageous for systemic gene therapy approaches. Transduction of cell types other than the target can have detrimental effects and diminishes the impact and effectual dose of the vector. Targeted delivery to the appropriate cell type would remove these risks and allow lower vector doses to be administered. In addition, there are cells that are refractory to transduction owing to poor expression of the endogenous cellular receptor. Various attempts have been made with different vector systems to alter the host range (100, 101). Among the strategies that have been explored is the approach of pseudotyping with elements from different viruses. Chemical attachment of ligands has been used to impart specific binding characteristics to purified vector virions. Although cell-specific targeting can be achieved by this approach in vitro, the stability of the complexes may not be sufficient to achieve targeting in vivo. Direct genetic modification is an attractive approach to introduce targeting ligand peptides into viral proteins, which mediates binding with alternative cellular receptors. Often the major challenge is to maintain high infectivity for modified vectors.

Retroviruses have been retargeted by fusion proteins of the envelope with single-chain variable antibody fragments to form a bispecific protein that binds to a specific cell surface protein (102). Ligands have also been introduced as extensions to the native surface (SU) glycoprotein (103). Although these vectors have demonstrated altered tropisms, they tend to exhibit low levels of infectivity.

Recombinant adenoviruses have been generated from a number of different serotypes, and these show variations in cell tropism (104). Pseudotyping with different fiber proteins is sufficient to alter tropism (105, 106). Viruses have also been engineered to express chimeric fiber proteins (107). Targeted Ad vectors are likely to hold more promise for systemic gene delivery and should possess reduced immunogenicity and toxicity, and hence increased safety (108). Bispecific proteins have been generated to retarget the vector to a number of cell types by fusing antibody fragments to the virus (normally to the fiber knob) together with targeting ligands (109). Targeting peptides have also been introduced directly into the fiber knob. Increased transduction of a number of cell lines devoid of high levels of the CAR was demonstrated with viruses containing fiber fused to RGD (110) or polylysine sequences (111). Nontarget transduction can be reduced by ablating the natural binding to the CAR and integrin receptors (112).

The simplicity of the structural elements of the AAV capsid makes it an attractive vector system for retargeting (113). Although AAV2 displays a broad host range, certain cell types are resistant to AAV2 infection, probably owing to the lack of appropriate receptors. Differences in cellular tropism have been observed for viruses using the capsid proteins from serotypes other than AAV2, which use alternative receptors for cell attachment and entry (114–116). AAV1 capsids show the highest transduction efficiency in muscle and liver, whereas AAV5 displays high tropism for retina (115, 117, 118). Furthermore, AAV5 is able to transduce airway epithelia cells (119), and AAV3 shows tropism for hematopoietic stem cells (120), which are resistant to transduction by AAV2. Even with a single organ there may be differential transduction of specific cell types (116). Mixing different capsid proteins suggests that it is possible to generate chimeric vectors that impart novel transduction phenotypes compared to either parental capsid alone (121). Sequence analysis of the cap proteins reveals regions with considerable diversity that are likely to be responsible for different tropisms (59). Altering the tropism of AAV vectors has also been explored by chemical cross-linking of bispecific antibodies to the viral capsid (122) and by the insertion of receptor-specific epitopes in the cap proteins (123–126). Library approaches may allow the most efficient targeted vector to be selected from a pool of viruses containing ligands, after transduction of the cell type of choice (127, 128).

VECTOR RECOGNITION, PROCESSING, AND INTEGRATION

The recognition and processing of vector genomes will be important for determining the efficiency of transgene expression and, therefore, ultimately the success of the gene therapy approach. Integrating vectors provide a means to achieve long-term gene expression, assuming that there is no silencing of transcription from the inserted DNA. Associated with integration into the host cell genome will be the risk of disrupting an essential gene or misregulating transcription. Insertional mutagenesis by vector DNA has long been recognized as a potential hazard of gene therapy, and recent clinical studies have highlighted this danger. Although many of the vector systems being used do not integrate, the delivered transgene can be expressed over long periods of time from vector DNA that persists extrachromosomally. Processing by cellular machinery can produce structures that are stably maintained, and elements can also be rationally incorporated into vectors to promote episomal maintenance.

For retrovirus infections, integration into the host genomes is a necessary step in the virus life cycle (129, 130). It occurs with high efficiency but without clear preference for specific target sequences or loci. The viral integrase protein mediates the joining reaction, with cellular DNA repair proteins also implicated at various stages (131). The precise mechanisms used to determine the selection of the integration remain unknown. Until recently it was thought that retroviral integration occurred randomly and that the risks of cancer induction from a single retroviral integration event was negligible. The availability of the human genome sequences has enabled genome-wide analysis of integration site usage for different retroviruses (132). These studies have revealed that different retroviruses may have quite varied preferences for integration sites in human chromosomes. HIV strongly favors active genes (133), whereas MLV favors integration near transcription start regions and only weakly favors active genes (132). In contrast, the avian sarcoma-leukosis virus shows only a very weak bias toward integration in active genes and no favoring of integration near transcription start sites. These observations suggest that integration site selection is affected by different interactions at the integration site and that tethering through protein-protein interactions may play a role. This raises the possibility that a particular safe site within the genome might be favored for integration through fusion proteins that tether the integrase to a specific target sequence (134).

AAV vector dsDNA genomes can persist in transduced cells for long periods of time by either integration or extrachromosomal maintenance. The frequency of rAAV integration is quite low (135, 136), and therefore, the associated risks may be much reduced when compared with retrovirus vectors. The wild-type AAV virus has a unique ability to integrate its DNA in a site-specific manner into a locus on human chromosome 19 (57). Although this has been shown in vitro, there is no evidence for site-specific integration from human samples. Targeted integration requires the viral Rep protein, and as this is deleted from most rAAV vectors, the targeting is lost. Recent genome-wide studies with rAAV vectors have shown a preference for integration into genes compared to noncoding regions in vivo (136). Although the precise mechanism of rAAV integration is unclear, it involves cellular pathways of recombination and may take place at naturally occurring chromosomal breakage sites (135).

Recombinant AAV vectors can also be detected in high-molecular-weight DNA molecules that are thought to represent concatamers formed by head-to-tail recombination of the ITRs. Inside the nucleus of infected cells, the rAAV genomes are somehow recognized and processed into circles and concatemers that can continue to express the transgenes for long time periods (137). Although it is unclear which cellular factors are involved in the process, studies in severe combined immunodeficiency (SCID) mice suggest that repair proteins may influence the episomal structure of rAAV genomes and circle formation (138, 139). There is also evidence that cellular DNA repair proteins can associate directly with the AAV ITR, and their relative binding may influence processing and transduction (140).

Although the genomes of Ad and HSV-1 are not normally integrated during virus infection, engineering of viruses can promote persistence of the vector genomes. For example, incorporation of elements from the Epstein-Barr virus (*oriP* and EBNA-1 protein) has been demonstrated to aid maintenance of the viral DNA and prolong transgene expression in both Ad and HSV-1 amplicon vectors (141, 142). Large DNA viruses can also be used as carriers for delivery of integrating viruses in hybrid vector systems. The targeting ability of the Rep protein of AAV can be harnessed for site-specific integration from hybrid vector systems that contain a transgene flanked with AAV ITRs. Hybrids of AAV elements within Ad (143, 144) and HSV-1 (145, 146) vectors have been developed with this goal in mind. In addition, integration into specific predetermined sites may also be achieved with other virus-derived recombinases, such as ΦC31 bacteriophage (147).

Gene Regulation

For gene therapy to become a successful modality of modern medicine, it will be necessary to regulate expression of the transgene to the appropriate level. Most vector systems utilize strong constitutive viral promoters to achieve high levels of transgene expression. However, systems have been developed that utilize tissue-specific transcriptional regulation or regulatable transcriptional elements that can be switched on and off via exogenous stimuli. Tissue-specific promoters are often much weaker than the heterologous viral promoters, but transgene expression can also be controlled at the level of translation by inclusion of *cis*-acting post-transcriptional regulatory elements (PREs). The PREs most commonly used are derived from viral transcripts, and the PRE from the woodchuck hepatitis virus has been shown to increase reporter gene expression at least fivefold in viral vectors (148, 149).

The regulatable systems used in viral vectors are either based on naturally occurring inducible promoters that exhibit tissue specificity or consist of chimeric systems engineered from various prokaryotic or eukaryotic elements (see Reference

150 for a review). Among the chimeric regulatable systems, the tetracycline (tet) regulatable system (151) is one of the best characterized and most widely used systems. It is based on binding of the tet repressor of *Escherichia coli* to the tet operator sequence and has been developed to give transactivation in the presence or absence of tet (152). Single-vector systems have been developed to deliver the transgene under control of the inducible promoter together with the gene encoding the regulator (153–155).

Another family of chimeric regulated systems is based on steroid hormones and their nuclear receptors. Systems have been developed on the basis of hormones and their receptors from insects and mammals. The progesterone system is based on a mutated human progesterone receptor, which is activated by the antiprogesterone mifepristone (RU486) but not the endogenous molecule (156). The use of the insect ecdysone-responsive systems receptor system has the potential advantage that the ecdysone hormones are neither toxic nor known to affect mammalian physiology. Systems based on both the *Drosophila* and *Bombyx* ecdysone receptors have been developed (157, 158) and shown to function in viral vectors.

Chemically induced dimerization can be used as a way to control the activity of a bipartite transcription factor (159). Inducible dimerization based on binding to rapamycin has been used to regulate transcription in retroviral vectors (160), with low basal expression levels and high dose-dependent induction of transgene expression. In vivo regulation of gene expression using this inducible system has also been demonstrated after intramuscular injection of AAV vectors (161, 162).

Another means of regulating gene expression is through the development of designer transcription factors. Modular DNA-binding protein domains can be assembled to recognize a given sequence of a DNA in a regulatory region of a targeted gene (163). Transcription factors can then be engineered by linking the DNA-binding protein to a variety of effector domains that mediate transcriptional activation or repression. Zinc-finger protein transcription factors (ZFP-TFs) have been designed to control the expression of any desired target gene and, thus, provide potential therapeutic tools for the study and treatment of disease. For instance a ZFP-TF can repress target gene expression with single-gene specificity within the human genome. HIV-1 transcription and genes, including Chk2, ErbB2, and VEGF, have been regulated by this approach (164).

Unfortunately, there are no gene regulatory systems that have yet received U.S. Food and Drug Administration (FDA) approval for clinical use. In addition to these approaches for regulating gene expression, the emerging small interfering RNA technologies are likely to become an integral component of successful gene therapy strategies.

CLINICAL TRIALS: SUCCESSES AND SETBACKS

Worldwide, over 600 clinical trials using gene therapy have been conducted or are underway, with the enrollment of thousands of patients (see Figure 3). A substantial portion of these trials (over 70%) are cancer related and are often carried out using

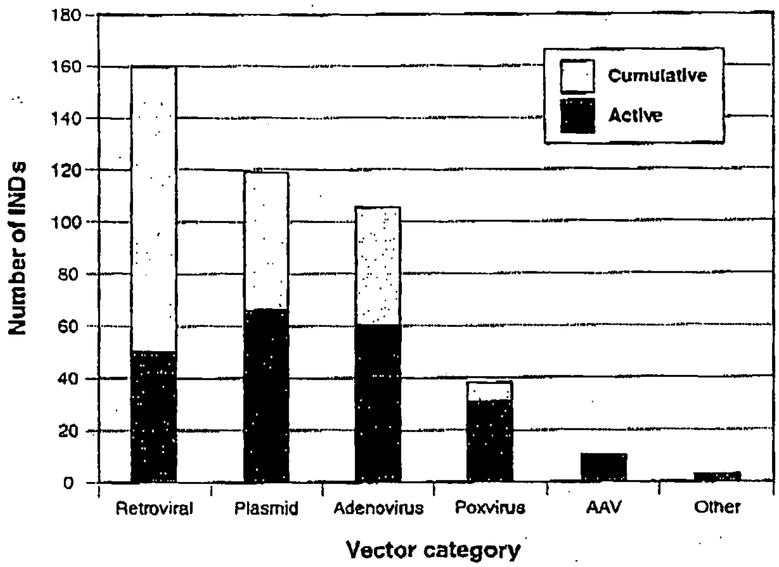

Figure 3 Clinical trials in gene therapy. A breakdown of the number of Investigational New Drug Applications relating to gene therapy as of March 2004, categorized according to the mode of delivery.

terminal patients. Most of the clinical trials are in Phase I or II, with less than 1% in Phase III, and therefore, there are presently no commercially approved gene therapy treatments.

Gene transfer into multipotent hematopoietic stem cells has received much attention because of its relevance for a broad variety of human diseases, ranging from hematological disorders to cancer (165). Retroviral vectors based on MLV were the first viral vectors to be used in a gene therapy trial and continue to be the most used. The first clinical trial attempted to treat SCID patients suffering from adenosine deaminase (ADA) deficiency, using retroviral vectors to transduce T lymphocytes (166). This study failed to show a long-term beneficial effect even though long-term reconstitution from transduced progenitor cells was observed at low levels. In addition to the transduced cells, these patients received ADA enzyme preparations, which might have prevented the selective outgrowth of the transduced progenitor cells. Although T-cell counts and function remained within normal limits, a loss of B and natural killer (NK) cells was observed in these patients, and ADA production was insufficient, probably owing to the limited number of transduced cells or low ADA expression levels.

The most successful trial that caught the attention of practioners of gene therapy and the general media was by Alain Fischer on children suffering from a fatal form of SCID, SCID-X1. This disease is an X-linked hereditary disorder characterized by an early block in the development of T and NK cells because of mutations in the γc cytokine receptor subunit. Hematopoietic stem cells from patients were stimulated and transduced ex vivo with an MLV-based retroviral vector, expressing the γc cytokine receptor subunit, and then were reinfused into the patients (167). During a 10-month follow up, γc-expressing T and NK cells could be detected, and cell counts and function were comparable to age-matched controls. The selective advantage of the γc-expressing lymphocyte progenitors contributed considerably to the success of this study. Unfortunately, three years after therapy was completed two of the children developed T-cell leukemia (168). In blood samples from these children, the leukemic cells contained a single intact copy of a retroviral vector that had integrated in or near the LMO2 gene (169). Although the precise mechanism by which LMO2 activation may cause T-cell leukemia remains conjectural, it is likely that the event is triggered by retroviral vector insertion. These results have rekindled the debate on the safety of gene therapy vectors.

The development of lentiviral vectors, which are able to transduce a hematopoietic progenitor in the absence of cytokines, might further improve stem cell gene therapy. Although these vectors have not yet been approved for use in clinical trials, some remarkable results have been obtained in animal models. Lentiviral vectors were successfully used to introduce a functional β-globin gene into hematopoietic stem cells and corrected β-thalassaemia and sickle-cell disease in mice models (170, 171). Furthermore, lentiviral vectors hold great promise in the treatment of neurological diseases, as demonstrated in a rhesus monkey model for Parkinson's disease (172) and a mouse model for metachromatic leukodystrophy, a lysosomal storage disease affecting the central nervous system (173).

In another clinical study, patients suffering from hemophilia B, a bleeding disorder caused by a deficiency of coagulation factor IX, were treated with AAV vectors expressing human factor IX (174). These patients participated in a Phase I trial and received intramuscular injections of AAV vectors. Although only very low levels of secreted factor IX could be detected in the plasma of one patient, the treated patients showed some clinical benefits and a reduced intake of factor IX infusions. Moreover, no vector-related toxicity and germline transmission was observed. These trials have now been abandoned in favor of injecting AAV factor IX vectors directly into liver, which in turn have shown some unexplained toxicity.

One of the earliest hopes of gene therapy approaches was the possibility of using viral vectors to either introduce a lethal gene to cancer cells or to boost the immune system so as to recognize the tumor cell as foreign [some approaches and progress are reviewed in (175, 176)]. In addition to using viruses to deliver tumor suppressors, apoptotic inducers, suicide genes, and cytokines, another attractive approach to cancer gene therapy is to harness the lytic action of replicating viruses for tumor-specific killing (177). Viral oncolysis can be achieved with RNA viruses and also with DNA viruses such as Ad and HSV-1 (178–180). Restrictive growth in cancer cells can be combined with delivery of a therapeutic or toxic gene, and in addition, the immunogenic properties of Ad vectors can elicit an indirect antitumor effect. Promising results have emerged from Phase II clinical trials using intratumoral administration of oncolytic viruses in combination with standard chemotherapy.

Clinical trials involving gene therapy are closely monitored, and any adverse event is extensively scrutinized. In 1999, the University of Pennsylvania initiated a human Phase I clinical trial for the treatment of patients with deficiencies in ornithine transcarbamylase. This trial was designed to test the safety of an E1/E4-deleted recombinant adenovirus vector (181). An 18-year-old volunteer in the study, Jessie Gelsinger, received the highest dose and four days later became the first person to die as a result of vector delivery (182). This was heralded as a death of gene therapy, but as in many experimental therapies, it is hard to anticipate unexpected pitfalls. The community of gene therapy has learned some valuable lessons from this tragedy, particularly the value of reporting all adverse events and clear definition of clinical end points. As a result of this tragedy, there is also better coordination between regulatory agencies.

PERSPECTIVES: WHAT IS NEXT?

Rarely comes along a modality of medicine that has the prospect of such widespread and profound influence on human health. The young field of gene therapy promises major medical progress toward the cure of a broad spectrum of human diseases, ranging from immunological disorders to heart disease and cancer. It has, therefore, generated great hopes and great hypes, but it has yet to deliver its promised potential. The idea to use the genetic information obtained by sequencing of the human genome for the treatment of diseases is compelling. However, if scientists from many different disciplines participate and pull together as a team to tackle the obstacles, gene therapy will be added to our medicinal armada and the ever-expanding arsenal of new therapeutic modalities. Geneticists are required to identify target genes that contribute to specific diseases or to identify those that can influence the course of disease. The task for virologists is to develop efficient and safe vectors that are able to deliver the genes of interest to the target cells and that assure the proper expression of the transferred genetic material. Cell biologists will establish ways to facilitate the gene transfer and will identify stem cells that may be used to regenerate failing organs. Bioengineers are needed to show the biologists how three-dimensional tissues and even whole organs may be generated in a test tube. Clinicians will ultimately carry out clinical trials with vectors optimized for the disease and for the medical requirements of the patients.

Gene therapy has suffered highs and lows in both the public and scientific communities. It has undergone extreme scrutiny in the recent past. It is our responsibility to assure the public that the patients' welfare and health is the major goal. Strict adherence to the guidelines is incumbent on all scientists and investigators involved in a clinical trial. The gene therapy community will need to meet the challenge of new regulations and guidelines introduced by the National Institutes of Health (NIH) and FDA to ensure both the quality of clinical trials and protection of volunteers enrolled in the trials. Together researchers and clinicians will be able to continue to participate and lead the nation in harnessing natural biological processes to provide real therapeutic benefits in this unprecedented golden age of biomedical research.

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